EVALUATION OF ROLE OF XPERT MTB/RIF IN DIAGNOSIS OF EXTRA PULMONARY TUBERCULOSIS IN PATIENTS ATTENDING TERTIARY CARE HOSPITAL

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Branch – IV

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Certificate

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CERTIFICATE –I

This is to certify that the dissertation work entitled **"Evaluation of role of Xpert MTB/RIF in diagnosis of extra pulmonary tuberculosis in patients attending tertiary care hospital"** submitted by **Dr. C. Ashmi**, is work done by her during the period of study in this department from September 2017- September 2018. This work was done under the guidance of **Dr. B. Appalaraju,** Professor and Head, Department of Microbiology, PSGIMS&R.

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CERTIFICATE- II

This is to certify that this dissertation work titled **"Evaluation of role of Xpert MTB/RIF in diagnosis of extra pulmonary tuberculosis in patients attending tertiary care hospital"** of the candidate **Dr. C. Ashmi** with registration number **201614402** is for the award of the degree **M.D. Microbiology, Branch IV**. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **6**% of plagiarism in the dissertation.

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"Evaluation of role of Xpert MTB/RIF in diagnosis of extra pulmonary tuberculosis in patients attending tertiary care hospital"** is a bonafide and genuine research work carried out by me under the guidance of Dr. B. Appalaraju, Professor and Head, Department of Microbiology, PSG IMS&R, Coimbatore. This dissertation is submitted to The Tamil Nadu Dr. M.G.R Medical University in fulfilment of the university regulations for the award of MD degree in Microbiology. This dissertation has not been submitted for award of any other degree or diploma.

Signature of the Candidate

Dr. C. Ashmi

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Introduction

INTRODUCTION

India is a country with one fourth of the Global Tuberculosis burden. The statistics for 2015 by World Health Organisation says that out of 9.6 million global incidence of tuberculosis, India accounts for about 2.2 million cases. And among the 2.5 million prevalence of tuberculosis 4.8 lakh people died of tuberculosis. Thus India ranks high in the Global burden of tuberculosis, causing mortality and morbidity worldwide. [1]

According to WHO report 2016, 1.3 million and 374000 tuberculosis death were reported among HIV negative and HIV positive people respectively. Around 10.4 million people fell ill with tuberculosis and among them 90% were adults, 65% were male and 10% were people with HIV. Among children 1 million fell ill with tuberculosis and 250000 children died due to tuberculosis. Over 95% of deaths due to tuberculosis, found to occur in low and middle income countries. One of the health targets of sustainable development goals by WHO is to end tuberculosis epidemic by 2030. [2] It is estimated that nearly one third of the population is latently infected with tuberculosis. Incidence of both pulmonary and extra pulmonary tuberculosis increases with the co -existence of HIV infection. In spite of its high global burden, the detection rate is very low, which poses major hurdle for tuberculosis control. And tuberculosis control is further complicated by the emergence of multidrug resistant tuberculosis. [3-5]

Tuberculosis can manifest as pulmonary or extra pulmonary tuberculosis. When it affects body sites other than lungs such as lymph node, meninges, kidney, spine, growing end of bones, it is known as extra pulmonary tuberculosis. Globally extra pulmonary tuberculosis (EPTB) accounts for 25% of all tuberculosis cases. But in

immunocompromised individuals, such as HIV infected and children, the percentage increases to even 50%. In India 20% of all tuberculosis cases are attributed to extra pulmonary tuberculosis with about 30-53% of extra pulmonary tuberculosis cases in tertiary care centres. [6] Major portion of the extra pulmonary tuberculosis is contributed by pleural tuberculosis which occurs in up to 30% of patients concomitantly affected by pulmonary tuberculosis. [7]

In the current scenario, diagnosis of EPTB is a diagnostic challenge as it involves invasive procedures, special expertise to carry out the procedure, paucibacillary nature of the specimen and non- specific signs and symptoms. The demonstration of TB bacilli by staining or culture is difficult, however histology can be suggestive. [8] The tests are also less accurate and time consuming. Conventional technique of smear microscopy had low sensitivity on these extra pulmonary samples and lead to higher false negative rates. Thus EPTB is easily misdiagnosed. [9, 10] Culture of *Mycobacterium tuberculosis* is the gold standard technique, but it also requires trained laboratory personnel and the absence of *Mycobacterium tuberculosis* in the given sample can be confirmed only after 8 weeks. This delay in diagnosis can cause more harm to the patients, so the treatment for *Mycobacterium tuberculosis* is often started empirically. [11, 12] The various clinical criteria and biochemical tests to confirm tuberculosis is less specific and sometimes tuberculosis is over diagnosed and treated with anti- tubercular drugs, which again lead to emergence of drug resistant *Mycobacterium tuberculosis*. [8] The search for a tool that can overcome the dilemmas of the diagnostic test available still continues.

The World Health Organisation recently implemented the light emitting diode (LED) fluorescent microscopy and the Gene Xpert MTB/RIF assay for National Tuberculosis Programme in developing countries. Compared to the conventional fluorescence microscopy, LED fluorescent microscopy is less expensive and has a sensitivity of 84% and specificity of 98% against culture as reference standard. [13] The Gene Xpert MTB/RIF assay (cartridge based nucleic acid amplification test) is a newly developed, automated diagnostic molecular test. This assay is based on nested real time PCR and molecular beacon technology with a sensitivity of detecting 131 cfu /ml of *Mycobacterium tuberculosis* in sputum or 4.5 genomes per reaction and simultaneous detection of rifampicin resistance mutations (81 bp region of rpo B gene) within two hours along with a sample preparation time of about 15 min. The rpo B gene is used as a surrogate marker for diagnosing multidrug resistant tuberculosis (MDR-TB). This Gene Xpert MTB/RIF assay requires minimal biosafety facilities as it is not prone for cross contamination. Patients with high risk of tuberculosis such as presumptive HIV associated tuberculosis patients and pediatric patients including extra pulmonary cases with negative smear for acid fast bacilli are mostly benefited from Gene Xpert assay. [14-16] The Gene Xpert MTB/RIF assay was initially evaluated only for pulmonary samples in large studies. It showed a sensitivity of around 89% and specificity of around 99% in pulmonary tuberculosis detection. But its use in detection of *Mycobacterium tuberculosis* in extra pulmonary samples is considered “off label” use. And its effectiveness in diagnosing extra pulmonary tuberculosis has not been demonstrated. [17] In order to improve the management of patients with suspicion of extra pulmonary tuberculosis the Gene Xpert MTB/RIF assay can be implemented in

both developed and developing countries. In the current situation it is not only important to detect tuberculosis cases but the determination of MDR status is also important. Therefore it is necessary to evaluate the Gene Xpert MTB/RIF assay for rapid diagnosis of extra pulmonary tuberculosis in routine laboratory use.

In India literature on the use of Xpert MTB/RIF for extra pulmonary samples is scarce. The purpose of the current study is to evaluate the performance of Xpert MTB/RIF assay for detection of *Mycobacterium tuberculosis* in extra pulmonary samples and to compare it with other conventional methods.

Aim and Objectives

AIM AND OBJECTIVES

AIM:

To evaluate Xpert MTB/RIF assay in diagnosing *Mycobacterium tuberculosis* in extra pulmonary samples.

OBJECTIVES:

1. To detect *Mycobacterium tuberculosis* in Ziehl Neelsen microscopy
2. To isolate *Mycobacterium tuberculosis* in Lowenstein Jensen culture medium
3. To isolate *Mycobacterium tuberculosis* in liquid culture medium (MGIT)
4. To rapidly identify *Mycobacterium tuberculosis* by Xpert TB/RIF
5. To find out the sensitivity and specificity of Xpert MTB/RIF over smear and culture.

Review of literature

REVIEW OF LITERATURE

HISTORICAL PERSPECTIVE:

Tuberculosis is a disease of ancient past. Hippocrates in 4000 BC gave a detailed account on the clinical features of respiratory and spinal tuberculosis. Tuberculosis is also referred to as rajayakshma (the king of diseases) in Vedas. Villemin in 1868 demonstrated the transmissible nature of tuberculosis by inoculating tuberculous material into rabbits and also recognised that tuberculous lymphadenitis and pulmonary tuberculosis were manifestations of the same disease process. [18]

The cause of tuberculosis was discovered in 1882 by Robert Koch. Then in the same year he also published an article titled "The Etiology of Tuberculosis". Koch's postulates became the basics for the study of all infectious disease. He observed the bacillus in association with all cases of disease, had grown the organism outside the host, and had reproduced the disease in a susceptible host.

Diagnosis of tuberculosis was supported mainly by the discovery of acid fast nature of bacillus by Ehrlich in 1882. Avian bacillus was recognized by Nocard in 1885. In 1890, Robert Koch discovered tuberculin, a substance derived from tubercle bacilli, but it turned out to be ineffective in arresting bacterial development. Later tuberculin proved to be a useful diagnostic tool. Nobel Prize in medicine was awarded for Robert Koch in 1905 for his contribution to tuberculosis. Theobald smith in 1898 differentiated bovine and human tubercle bacilli, which further lead to the identification of Non Tuberculous Mycobacteria. Later in 1907-1908, Von Pirquet and Mantoux developed the tuberculin skin test, which was once a useful diagnostic tool. Seibert in 1931 prepared a purified protein derivative of tuberculin. [19]

Robert Koch cultivated tubercle bacilli on inspissated serum and stained it with alkaline methylene blue for 24 hours. Further this staining technique was improved by Ehrlich by using carbol fuchsin and this was subsequently modified by Ziehl Neelsen, which is used widely today. [18]

GENERAL CHARACTERISTICS:

Mycobacterium tuberculosis complex:

Mycobacterium tuberculosis complex includes:

- *Mycobacterium tuberculosis*
- *Mycobacterium bovis*
- *Mycobacterium caprae*
- *Mycobacterium africanum*
- *Mycobacterim microti*
- *Mycobacterium pinnipedi*
- *Mycobacterium canetti*

Among all, *Mycobacterium tuberculosis* is the most common cause of tuberculosis in man. *Mycobacterium bovis* causes tuberculosis in warm blooded animals. Human disease by *Mycobacterium bovis* is similar to the one caused by *Mycobacterium tuberculosis. Mycobacterium bovis* strains are inherently resistant to pyrazinamide. But *Mycobacterium bovis subsp. caprae* is susceptible to pyrazinamide. BCG strains of *Mycobacterium bovis* is still used for vaccine. Human tuberculosis is Africa is caused by *Mycobacterium africanum*. Physiological and biochemical properties of *Mycobacterium africanum* lies between *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *Mycobacterium microti* is isolated from rodents. Their

morphology in stained smear is similar to a “croissant”. Lymphadenitis in children and generalized tuberculosis in HIV patients is caused mainly by *Mycobacterium canetii*. It produces glossy colonies which can be even confused with Non Tuberculous Mycobacteria (NTM). They are more abundant on the African continent. *Mycobacteium pinnipedii* is found in pinnipeds, pigs, rabbits, cattle and humans. Their infection usually manifests as granulomatous lesions. [18]

SCIENTIFIC CLASSIFICATION:

Kingdom: Bacteria

Phylum: Actinobacteria

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: Mycobacterium

Species: tuberculosis [18]

Acid fastness:

Mycobacterium tuberculosis resists decolorization by dilute mineral acids. This acid fastness character is mainly due to the presence of mycolic acids in the cell wall and integrity of cell wall.

Guanine and cytosine:

Guanine + cytosine content of DNA of *Mycobacterium*: 61-71 mol% [20]

HABITAT:

Mycobacterium species were isolated from environmental sources such as water, soil and dust. They can be classified as pathogens or free living saprophytes. [18]

MORPHOLOGY:

Mycobacteria are weekly gram positive, non-motile, non-sporing and non-capsulated. They are straight or slightly curved rod shaped bacteria, which are obligate aerobes. In liquid medium they sometimes show branching filamentous form.

ANTIGENIC STRUCTURE:

Antigens of *Mycobacterium tuberculosis* are of 2 types:

- Cell wall antigens
- Cytoplasmic antigens

Cell wall antigens:

Cell wall consists of distinct layers:

Peptidoglycan layer maintains the shape and rigidity. Plasma membrane is found beneath the cell wall. Survival inside the macrophage is mainly due to the arabinogalactan layer. The acid fastness of *Mycobacterium tuberculosis* is mainly due to mycolic acid. The outermost layer consists of lipids, glycolipids and mycosides. Porins are found throughout the various layers of cell wall.

Cytoplasmic antigens:

These include antigen 5, 6 and 60. They are used in serodiagnosis of tuberculosis. [20]

CULTURAL CHARACTERISTICS:

Tubercle bacilli are slow growing and they have a long generation time of 10-15 hrs. *Mycobacterium tuberculosis* is an obligate aerobe. Optimum temperature required for their growth is 37 ^{0}c. *Mycobacterium tuberculosis* shows eugonic growth whereas *Mycobacterium bovis* shows dysgonic growth on culture medium.

The colonies usually appear in about 2 to 8 weeks. When the culture media is added with 0.5 % glycerol, the growth of *Mycobacterium tuberculosis* is improved but this does not occur in case of *Mycobacterium bovis*. Para nitro benazoic acid inhibits the growth of *Mycobacterium tuberculosis* whereas *Mycobacterium bovis* is not inhibited by para nitrobenzoic acid. They are highly susceptible to even traces of fatty acids. The toxicity is neutralized by serum albumin or charcoal. Both solid and liquid media are described for *Mycobacterium tuberculosis.*

Solid media:

Egg based media:

- Lowenstein Jensen medium
- Petragnani and Dorset egg medium

Blood based media:

- Tarshis medium

Serum based media:

- Loeffler medium

Potato based media:

- Pawlowsky medium

Agar based media:

- Middlebrook 7H11 medium
- Middlebrook 7H10 medium

Liquid media:

- Middlebrook 7H9 medium
- Dubos medium

- Proskauer medium
- Sula medium
- Sauton medium

Liquid media are not employed for routine culture. They are mainly used for drug sensitivity testing and preparation of antigens and vaccines.

IDENTIFICATION OF BACTERIA:

The first step to confirm that the isolate is an acid fast organism is by performing an acid fast staining. Colony morphology, growth rate, optimum temperature and photo reactivity helps in the speciation of *Mycobacteria.*

Colony appearance:

Rough, dry, creamy white, buff coloured and raised colonies with irregular wrinkled surface were seen on solid culture. In liquid media they are dispersed in the media, but virulent strains from long serpentine cords.

Growth rate:

Mycobacteria are usually classified as slow growers and rapid growers. Rapid growers produce colonies in less than 7 days.

Temperature:

The growth of *Mycobacterium tuberculosis* occurs at 37^0 c.

Photo reactivity:

According to photo reactivity, *Mycobacterium* has been grouped into 3 groups.

- Photochromogens:

  Produce pigment upon exposure to light

- Scotochromogens:

Produce pigment in light or dark

- Non photochromogens:

  On exposure to light does not produce pigment [21]

BIOCHEMICAL IDENTIFICATION: [21, 22]

Several biochemical tests were available for *Mycobacterium* species identification. They were Niacin test, Aryl sulphatase test, Catalase peroxidase test, Nitrate reduction test, Amidase test, Pyrazinamidase test, Inhibition by thiophene-2 carboxylic acid, Neutral red test and Tween 80 hydrolysis.

Niacin test:

When grown on egg medium, *Mycobacterium* forms niacin. 10% cyanogen bromide and 4% aniline in 96% ethanol, when added to the culture, a canary yellow colour indicates a positive reaction. In this test, nicotinic acid reacts with cyanogen bromide in the presence of an amine to form a yellow pigmented compound. The test is positive with *Mycobacterium tuberculosis* and negative with *Mycobacterium bovis.*

Aryl sulphatase test:

The bacilli are grown in media containing 0.001 M tripotassium phenolphthalein disulphate. To this, 2N NaOH is added drop by drop. Pink colour formation indicates positive reaction, which is given by atypical mycobacteria.

Catalase- peroxidase test:

This test helps not only to differentiate tubercle bacilli from atypical mycobacteria, but also indicates the sensitivity of the strains to isoniazid. Catalase is an intracellular enzyme that can split hydrogen peroxide into water and oxygen. A mixture of 30 vol. hydrogen peroxide and 0.2% catechol in distilled water is added to

5 ml of the test culture and then allowed to stand. Catalase production is indicated by effervescence and peroxidase activity is indicated by browning. Tubercle bacilli are peroxidase positive. Both catalase and peroxidase activities are lost when the tubercle bacilli becomes isoniazid resistant.

Nitrate reduction test:

To the suspension of bacteria sulphanilamide and n- naphthyl- ethylene is added. Nitroreductase catalyses the reduction of nitrate to nitrite, a red colour is formed, which indicates positive reaction. This test is positive for *Mycobacterium tuberculosis* and negative for *Mycobacterium bovis.*

Amidase test:

Mycobacterium can be differentiated by its ability to split amides namely acetamide, benzamide, carbamide, nicotinamide and pyrazinamide. To the bacterial suspension 0.00165M solution of amide is added and incubated at 37^0c and then 0.1 ml of MnSO4. 4H2O, 1 ml of phenol solution and 0.5 ml of hypochlorite solution are added to the suspension. Tubes are kept in boiling water bath for 20 minutes. Positive test is indicated by blue colour formation.

Pyrazinamidase test:

This test is positive in *Mycobacterium tuberculosis* and negative in *Mycobacterium bovis*. The pyrazinamidase enzyme hydrolyses pyrazinamide to ammonia and pyrazinoic acid which is detected by the addition of ferric ammonium sulphate. Pyrazinamidase negative *Mycobacterium tuberculosis* will be resistant to pyrazinamide.

Inhibition by thiophene-2 carboxylic acid:

This test differentiates *Mycobacterium tuberculosis* from *Mycobacterium bovis.* *Mycobacterium tuberculosis* is resistant (growth on thiophene -2 carboxylic acid is >1% of the growth in control).

Neutral red test:

This test differentiates virulent strains of tubercle bacilli from avirulent strains. Only virulent strains can bind neutral red in alkaline buffer solution.

Tween 80 hydrolysis:

This test is useful in differentiating *Non Tuberculous Mycobacteria* (NTM). Change in colour of the medium from yellow to red at pH 7 indicates a positive test.

PATHOGENESIS: [23]

T cell mediated immunity is the one that decides the outcome of tuberculosis infection in an immunocompetent person. Thus T cell controls the host response to mycobacterial infection. In early infection, *Mycobacterium tuberculosis* replicates inside the macrophages, while in late infection, macrophages are stimulated to contain the mycobacterial proliferation.

Entry into macrophages:

Once the *Mycobacterium tuberculosis* bacilli are inhaled, they are trapped in the upper airways and few bacilli reach the alveoli. Inside the alveoli, mannose binding lectin and CR3 helps in the entry of *Mycobacterium tuberculosis* into the alveolar macrophages. Complement activation leads to opsonisation of these bacilli and forms phagolysosome. The bacilli survive inside the phagolysosome by increasing

the intracellular calcium, by inhibiting the calcium / calmodulin complex using their glycolipid lipoarabinomannan. Thus the phagosome maturation is arrested and the macrophage is lysed to release the bacilli. [46] In the early stage of infection, the bacteria usually replicate in the alveolar macrophages and they are seeded to multiple sites to continue the infection cycle.

Initiation and consequences of cell mediated immunity:

Mycobacterium tuberculosis is recognised by toll like receptor -2 (TLR-2). Stimulation of TLR-2 promotes the production of IL-12 by dendritic cells. This IL-12 helps in the differentiation of T- helper 1 cells which further helps in the production of IFN- γ in both lymph nodes and lung. This IFN- γ helps to limit the mycobacterial infection by

- Phagolysosome maturation and activation
- Production of nitric oxide
- Production of reactive oxygen species and
- Autophagy.

TH1 response also causes the formation of granulomas (Epithelioid histiocytes). TNF (Tumour Necrosis Factor) and chemokines are secreted by activated macrophages helps in the recruitment of monocytes. Reactivation of tuberculosis is common in rheumatoid arthritis patients treated with TNF antagonists. People with IL-12 pathway deficiency and IFN- γ pathway deficiency are susceptible to severe mycobacterial infections.

MODE OF TRANSMISSION: [21]

Mycobacterium tuberculosis is commonly transmitted by droplet nuclei (by Coughing, sneezing or speaking).Patient presents with signs and symptoms of evening rise of temperature, sweating, weight loss, hemoptysis and dyspnoea.

Virulence of tubercle bacilli: [46]

Different patterns of virulence defects have been defined in animal models. The KatG gene encodes for catalase/ peroxidase enzyme which protects the bacilli from oxidative stress. Early secretory antigen-6 and culture filtrate protein- 10, encoded by region of difference 1 (RD1) is absent in BCG vaccine strain *Mycobacterium bovis*. The leu D and pan CD mutants become auxotrophic as they require leucine and pantothenic acid. The icl1 (isocitrate lyase gene) is required for bacterial growth on fatty acid substrates. The carD gene is required for replication and persistence in the host cells. So its loss leads to bacterial killing by defensive mechanisms.

CLINICAL MANIFESTATIONS:

Tuberculosis is classified as pulmonary and extrapulmonary tuberculosis. Based on the time of infection and type of response it is categorized as primary and post primary / secondary tuberculosis.

Primary tuberculosis:

Initial infection by tubercle bacilli localized to lower lobe or lower part of upper lobe. This is known as Ghon focus. The Ghon focus together with enlarged hilar lymph node constitutes the primary complex. In about 2- 6 months, the lesion heals spontaneously of remain latent. In case of immunocompromised individuals, the primary lesion may progress to disseminated infection.

Post primary/ secondary tuberculosis:

This occurs mainly due to reactivation of latent infection. Secondary tuberculosis usually affects the upper lobe, where there is high oxygen concentration for mycobacterial growth. The lesion undergoes necrosis, tissue destruction leading o cavitation. In immunocompromised persons cavity formation doesn't occur. [21]

Extrapulmonary tuberculosis: [46]

Extra pulmonary tuberculosis is more commonly seen today than in the past. Lymph nodes, pleura, meninges, peritoneum, genitourinary tract, bones and joints and pericardium are the most commonly involved sites in extra pulmonary tuberculosis.

Lymph node tuberculosis:

Lymph node tuberculosis is commonly seen among HIV infected patients. It usually presents as painless swelling of posterior cervical and supraclavicular lymph nodes. Initially the lymph nodes are discrete and then become matted. The cultures are positive in 70-80% of cases. Differential diagnosis for lymph node tuberculosis includes lymphomas, metastatic carcinomas, kikuchi's disease, kimura's disease and castleman's disease.

Pleural tuberculosis:

Pleural tuberculosis presents with fever, chest pain, dyspnea, dullness on percussion and absent breath sounds. The pleural fluid shows increased protein, low to normal glucose and detectable white blood cells. Only in 10-20% of cases acid fast bacilli is seen on direct smear and in 25-75% of cases the culture is positive for *Mycobacterium tuberculosis*. Adenosine deaminase is a screening test for pleural

tuberculosis. It may progess to tuberculous empyema following rupture of cavity and spillage of organisms in the pleural cavity.

Tuberculosis of upper airways:

It involves larynx, pharynx and epiglottis. Patients present with hoarseness, dysphonia, dysphagia and productive cough.

Genitourinary tuberculosis:

Genitourinary tuberculosis is more commonly seen in female than in male patients. The symptoms include urinary frequency, dysuria, nocturia, hematuria and flank pain. It is usually diagnosed by culture of three morning urine specimens. It responds well to treatment.

Skeletal tuberculosis:

The commonly affected bones are spine [61] (potts disease), hip and knee joint. Upper thoracic spine is commonly affected in children; lower thoracic and upper lumbar vertebrae are affected in adults. Paravertebral cold abscess can also be seen in skeletal tuberculosis.

Tuberculous meningitis and tuberculoma:

Tuberculous meningitis occurs as a result of hematogenous spread. It presents as headache, slight mental changes, low grade fever, malaise, anorexia and irritability. Diagnosis is by examination of CSF which reveals low glucose, high protein and high leucocyte count with predominant lymphocytes. Culture of CSF is the gold standard test and PCR has a sensitivity of 80%. Tuberculoma presents as space occupying lesion with symptoms of seizure and focal signs. Usually biopsy is required for diagnosis of tuberculoma.

Gastrointestinal tuberculosis:

Any portion of gastrointestinal tract can be involved, but ileum and caecum are the common sites affected by tuberculosis. The signs and symptoms include abdominal swelling, hematochezia, palpable mass in abdomen, fever, weight loss, anorexia and night sweats. Tuberculous peritonitis should be suspected when there is unexpected fever, ascites and nonspecific abdominal pain. Smear and culture yield of ascitic fluid is low, so biopsy is needed for diagnosis.

Pericardial tuberculosis:

It usually affects elderly population and HIV infected patients. It presents with dyspnea, fever, dull retrosternal pain, and pericardial friction rubs. The effusion is exudative with lymphocytes and monocytes. Direct smear of pericardial fluid is very rarely positive but culture is positive for *Mycobacterium tuberculosis* in two thirds of cases. Adenosine deaminase, IFN gamma assay, lysozyme and PCR [63] may be useful in diagnosis.

Miliary or disseminated tuberculosis:

It occurs due to hematogenous spread of tubercle bacilli. It can also be a consequence of primary infection, recent infection or reactivation of old foci. In 80% of cases the sputum smear microscopy will be negative. Miliary reticulonodular pattern is observed in chest radiography and on eye examination it shows choroidal tubercle. The patient usually presents with respiratory and abdominal symptoms. If not detected early, miliary tuberculosis is fatal.

Less common extra pulmonary forms:

- Chorioretinitis
- Uveitis
- Panophthalmitis
- Tuberculous otitis
- Scrofuloderma
- Lupus vulgaris
- Erythema nodosum

HIV associated tuberculosis:

Tuberculosis accounts as a major cause of death in HIV patients. [62] Extra pulmonary tuberculosis is more common among HIV patients. Tuberculosis can occur at any stage of HIV but the presentation varies. In early stage when the CMI (Cell Mediated Immunity) is partially compromised, tuberculosis presents in typical manner. In late stages of HIV infection, tuberculosis presents as diffuse interstitial or military infiltrates with minimal or no cavitation and intra-thoracic lymph node enlargement.

METHODS OF DETECTION OF *MYCOBACTERIUM TUBERCULOSIS*:

Microscopy:

A presumptive diagnosis is usually based on microscopic examination of the specimen. [46] It is the fast and inexpensive method to detect tuberculosis in high incidence areas. But only about 50% of the active tuberculosis cases are detected by smear microscopy. The smears can be stained by ziehl neelsen stain or

auramine/rhodamine fluorochrome dyes. Atleast 2 ml of purulent sputum should be collected as spot- spot specimen or spot- early morning specimen. Nearly 10,000 bacilli/ ml should be present to be detected by smear microscopy. And the sensitivity of this technique varies from 20- 80%. [24, 25] The use of smear microscopy is limited in urine or gastric fluid specimens due to the false positive reaction given by commensal *Mycobacteria*. [46]

Fluorescent microscopy with light emitting diodes:

LED microscopes are user friendly microscopes. They don't emit UV light; don't require darker rooms and the power consumption is also less with these microscopes compared to conventional microscopes. The smears are examined 4 times faster than the conventional microscopes. But the sensitivity and specificity of this technique has not been established. [26, 27]

Sodium hypochlorite/ bleach microscopy:

In this microscopy, prior to smear examination, the sputum sample is digested with sodium hypochlorite to increase the yield. This method have been developed and evaluated recently. [24]

Vital fluorescent staining:

Fluorescein diacetate is a fluorescent viability marker which is used in combination with smear microscopy. This technique is very useful in guiding antimicrobial treatment before the culture reports are ready. [28]

Newer techniques:

Automated microscopic technology and cellscope are the newer technologies available for smear microscopy. [28, 29]

Culture:

Definitive diagnosis of tuberculosis is based on the culture isolation and identification of *Mycobacterium tuberculosis*. [46] Culture on Lowenstein Jensen media (an egg based media) and Middlebrook 7H10/7H11 (liquid media) are available. In well-equipped laboratories, they use liquid culture for isolation of *Mycobacterium tuberculosis.* Even now culture remains the gold standard method. The detection limit of sputum culture is 100 bacilli/ml, but it takes around 8 weeks for the culture to be reported negative. And another 4 weeks is required for drug susceptibility testing. [30]Mycobacterial growth indicator tube (MGIT), a non-radiometric detection method. The fluorescent compound tris 4, 7- diphenyl- 1, 10- phenanthroline ruthenium chloride pentahydrate is embedded in the silicone coated round bottomed tubes.

When there is bacterial growth, actively respiring microorganism will consume the oxygen and the fluorescence will be evident in UV light. This fluorescence will be detected every 60 min for the growth of bacterium. Drug susceptibility can also be done in MGIT by 1% proportion sensitivity testing method. Negative culture report is issued in 42 days. The sensitivity and specificity of these culture methods are nearly 100%. [27, 31]

Rapid identification methods:

MPT64: strip speciation technique:

It is a rapid immunochromatographic lateral flow assay. [46]This test detects tuberculosis specific antigen MPT 64, which is a *Mycobacterium tuberculosis* complex specific antigen secreted during bacterial growth. The results are obtained within 15 minutes. This test cannot identify the MTB strains.

Antigen detection method:

Lipoarabinomannan (LAM) is detected by dipstick or ELISA method. LAM is found in the cell wall of *mycobacterium*. [32] Lateral flow LAM assay showed pooled sensitivity and specificity of 44% and 92% respectively. [27]

Molecular detection methods:

These molecular methods have utility in diagnosis of smear negative pulmonary tuberculosis and extra pulmonary tuberculosis. [47-49] Cartridge Based Nucleic Acid Amplification Test (CBNAAT), Line Probe Assay (LPA) and Loop Mediated Amplification (LAMP) tests are the molecular tests available for *Mycobacterium tuberculosis* detection. Both CBNAAT and LPA are recommended by World Health Organization. Molecular detection methods play a major role in diagnosing tubercular meningitis. The pooled sensitivity of these molecular tests for detecting tuberculosis from extra pulmonary samples range between 36% - 100% (85%) and the specificity range between 54% - 100% (97%). [25]

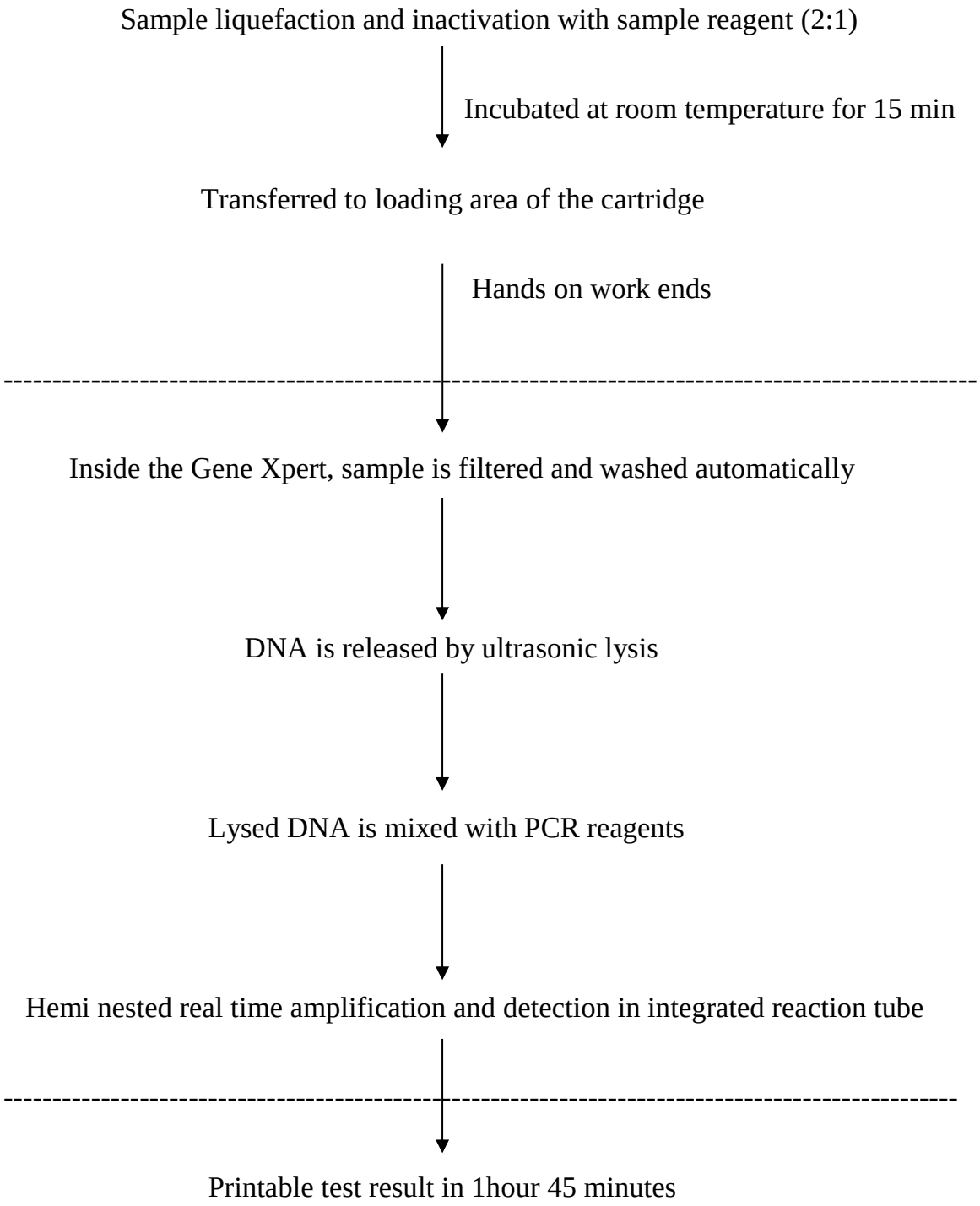
Cartridge based nucleic acid amplification test:

It is a WHO recommended molecular detection method for diagnosing pulmonary and extra pulmonary tuberculosis and for diagnosing paediatric tuberculosis. CBNAAT is a semi quantitative hemi nested real time PCR method. This cartridge based system uses microfluidics technology and fully automated nucleic acid analysis from clinical samples.[71] This assay uses single use plastic cartridges with multiple chambers which are already loaded with liquid buffers and lyophilised reagent beads for processing of samples. Gene Xpert cartridge consists of

- Syringe drive
- Rotary device
- Filter for *Mycobacterium tuberculosis* bacilli deposition
- Sonic horn at the cartridge base
- *Bacillus globigii* spores as internal sample processing and PCR control
- 5 overlapping molecular probes (A-E) - complementary to entire 81 bp rpo B core region. [71]

Here the specimen is processed and 2 ml of it is transferred to the loading area of the cartridge and it is inserted into the MTB-RIF platform. Inside the cartridge there occurs DNA lysis by ultrasonic vibration. And it uses 3 primers and 5 molecular probes to enhance specificity.

Steps in Gene Xpert Assay procedure:



Molecular beacon system:

The Xpert MTB/RIF assay uses molecular beacon technology to detect DNA sequences. [69, 70] The molecular beacons are nothing but an oligonucleotide sequence that contains a probe sequence inserted between 2 arm sequences. These arm sequence are complementary to each other and they form a stem- and- loop secondary structure after hybridization. Fluorophore and non-fluorescent quencher are linked to end of the two arms. The probe is situated within the loop structure. Each probe will be complementary to different target sequence within the rpo B gene and labelled with a differently coloured fluorophore. When the probe is in Free State or non-hybridized state, the quencher and fluorophore will be close to each other and the fluorescence is suppressed. However when it binds to DNA target, conformational change occur in the molecular beacon, which causes separation of the fluorophore and the quencher arms to separate thus causing bright fluorescence.[69] The rpo B gene 81 bp RIF resistance determining region is amplified to detect rifampicin resistance. CBNAAT have internal controls such as sample processing control and probe check control. CBNAAT detects both *Mycobacterium tuberculosis* and Rifampicin drug resistance within 2 hours. The detection limit of CBNAAT is 131 colony forming unit/ml. The analytical time of CBNAAT is around 2 hours to 3 hours. This test eliminated the problem of cross contamination. It detects both alive and dead bacilli.

Interpretation of Gene Xpert assay:

- Detection of *Mycobacterium tuberculosis*:

Mycobacterium tuberculosis is identified when two of the five probes show positive for *Mycobacterium tuberculosis* and a cycle threshold (C_T) of <= 38 cycles. If the internal control is negative for *Mycobacterium tuberculosis*, then the assay is invalid.

- Detection of Rifampicin resistance:

Rifampicin resistance is mainly detected by the difference between early cycle threshold range and late cycle threshold range.

$\Delta C_T > 3.5$ cycles - Rifampicin resistance

$\Delta C_T <= 3.5$ cycles - Rifampicin susceptible

First probe $C_T > 34.5$ and the last probe $C_T > 38$ - indeterminate [71]

Sensitivity and specificity of Gene Xpert:

The detection of smear negative pulmonary tuberculosis in paediatric age group by Gene Xpert showed a sensitivity of 33.3% and 61.1% when testing one or two samples respectively. [72] In case of adult the diagnosis of pulmonary tuberculosis by Gene Xpert showed a sensitivity of 94.7% in smear positive cases and 46.8% in smear negative cases. The overall specificity of this assay is around 78.7%.[73] Another study conducted in Tanzania showed a sensitivity of 98 % for smear positive pulmonary tuberculosis cases.[74] For non-respiratory samples Gene Xpert showed a sensitivity of 77.3% and specificity of 98.2%.[75] Another foreign study on detection of

Mycobacterium tuberculosis in non-respiratory samples by Gene Xpert MTB/RIF showed a sensitivity of around 95% and specificity of 100%.[76] Another study showed very low sensitivity of 53% for detecting *Mycobacterium tuberculosis* in extra pulmonary samples.[77] In India Gene Xpert evaluation for extra pulmonary samples showed a sensitivity of 81% and specificity of 99.6%. In case of smear positive specimens, the sensitivity is reported around 96% and in case of smear negative extra pulmonary tuberculosis specimens the sensitivity comes to around 64%.[78] In case of smear and culture positive cases the sensitivity is 99.8% and in smear negative and culture positive cases the sensitivity is 90.2%. CBNAAT detected Rifampicin (RIF) resistance with a sensitivity of 99.1% and specificity of 100%. [33, 34, 35, 36]

HIV associated tuberculosis:

This assay has increased the detection rate of tuberculosis in HIV patients from 20% to 45%. And the specificity of this assay is around 99.25 in HIV prevalent areas.[79] Rifampicin resistance detection in HIV associated tuberculosis by this assay reported sensitivity and specificity of around 96.8% and 96.2% respectively.[80]

Line Probe Assay:

LPA is a hybridization assay that differentiates *Mycobacterium species* and detects genetic mutations associated with drug resistance. It consists of a strip with 27 reaction bands including 6 controls (conjugate, amplification, *and Mycobacterium tuberculosis complex*, rpoB, katG and inhA controls), 8 rpoB WT, 4 mutants probe, 1 katG WT gene, 2 MUT probes, 2 inhA WT and 4 MUT probes. It takes around 5- 6 hours to provide the report. For rifampicin (RIF) resistance detection the sensitivity of

LPA is 98.9% and the specificity is 99.4%. For Isonazid (INH) resistance detection the sensitivity and specificity is around 98.8% and 100% respectively. Thus this test is approved for use in MDR tuberculosis patients by WHO. For detecting ofloxacin, amikacin and extensive drug resistance from clinical samples, the sensitivity of this test is 90.7%, 100% and 92.3% respectively, and the specificity is 98.1%, 99.4% and 99.6% respectively. In May 2016 WHO recommended this test to be performed for XDR tuberculosis patients also. [37, 38, 39]

Loop Mediated Amplification:

LAMP is an isothermal DNA amplification method that doesn't require a thermocycler. It mainly detects *Mycobacterium tuberculosis* complex but not the drug resistance. It is a simple, easy, rapid and cost effective DNA amplification method. The results will be available within 35- 60 minutes time. It uses 4 primers that targets at 6 different regions of the target gene. The detection limit is 5-15 copies of purified DNA. Other isothermal nucleic acid amplification tests include recombinase polymerase amplification, cross priming amplification, helicase dependent amplification and nicking enzyme amplification reaction.[40,41] The sensitivity of LAMP in smear positive and culture positive specimen is around 98.2%, while the sensitivity of smear negative and culture positive specimen is 55.6%. [67] Studies done in India showed the sensitivity of culture positive specimen as 100%, the specificity of culture negative sample as 94.2% and the positive and negative predictive value of LAMP as 94.1% and 100% respectively.[68]

Gene Xpert MTB/RIF Ultra:

Gene Xpert MTB/RIF Ultra is an advanced version of Xpert MTB/RIF with better tuberculosis detection and more definitive identification of Rifampicin (RIF) susceptibility and resistance. In this method two new PCR assays have been included that target 2 different multicopy genes. The rpoB and IS6110 assays have been converted into fully nested PCR. Silent mutations such as Q513Q and F514F associated with rifampicin resistance were detected in this method. Larger PCR tube is used in this test, which improves the sensitivity of detection of *Mycobacterium tuberculosis* H37Rv to 10 fold greater than the Gene Xpert MTB/RIF. The limit of detection of *Mycobacterium tuberculosis* H37Rv was 15.6 colony forming units /ml for sputum. [64] Gene Xpert MTB/RIF Ultra sensitivity for tuberculous meningitis was found to be 70% when compared with 43% for Gene Xpert MTB/RIF. Now a days for the detection of tuberculous meningitis WHO recommends the use of Gene Xpert MTB/RIF Ultra. [65]

Indirect methods:

Tuberculin skin testing/ Mantoux test:

Robert Koch in 1981 discovered that the components of *Mycobacterium tuberculosis* when injected subcutaneously into patients with tuberculosis produced skin reaction. Mantoux test was described by Mantoux and Von Priquetto. This test detects only presence or absence of tuberculosis. In Mantoux test, Purified protein derivative (PPD) of *Mycobacterium tuberculosis* is injected intradermally into the forearm and the reaction results are read after 48-72 hours. When the induration is 10

mm or more, it is considered as positive reaction. However vaccinated individuals can give positive reaction. [42, 43] This test is mainly used for screening f latent tuberculosis. The disadvantage of this test includes lack of species specificity, deterioration of product and batch to batch variations. [46]

Interferon gamma release assay:

It is an in vitro assay test for detecting latent tuberculosis that measures the T cell release of IFN- γ . The T cells on encountering the Mycobacterial antigens such as early secretory antigenic target -6 (ESAT-6), culture filtrate protein (CFP-10) and TB 7.7 causes the release of interferon gamma TH1cytokines from the T cells. Two assays are available, one is T SPOT- TB which is an enzyme linked immunospot assay and the other is QuantiFERON- TB Gold which is a whole blood enzyme linked immunosorbent assay for measuring IFN- γ . QuantiFERON-TB Gold assay also contains TB7.7 antigen. IGRA are more specific than tuberculin skin testing. [46] Here the T cells are sensitized with *Mycobacterium tuberculosis*. The antigen used is recognized by the T cells of tuberculosis patients not by BCG vaccinated or unvaccinated healthy individuals. These tests are not used for diagnosis of active tuberculosis infection.[44,45] The advantages of interferon-γ release assay include logistical convenience, few visits by patients to complete testing, and avoidance of subjective measurement of skin induration.[46]

TREATMENT OF TUBERCULOSIS:

The aims of tuberculosis treatment are

- To interrupt transmission of tuberculosis
- To prevent morbidity and mortality

The discovery of streptomycin in 1943 made the treatment of tuberculosis possible. Then in 1950 both para amino salicylic acid and isoniazid came into practice. With the introduction of rifampicin in 1970 led to effective short term treatment course. The four major drugs used for treatment includes Isoniazid, Rifampicin, Pyrazinamide and Ethambutol. [46]

Antituberculous drugs: [59]

- Isoniazid (H)
- Rifampin (R)
- Rifapentine
- Pyrazinamide (Z)
- Ethambutol (E)
- Streptomycin (S)
- Fluoroquinolones
- Bedaquiline

Second line agents:

- Ethionamide
- Cycloserine
- Terizidone
- Amikacin
- Kanamycin
- Capreomycin
- Thiacetazone
- Para- aminosalicylic acid (PAS)

Third line agents:

- Amoxicillin-clauvulanate
- Clarithromycin
- Clofazimine
- Linezolid

Agents under development:

- Oxazolidinones: sutezolid, AZD5847
- Nitroimidiazoles: delaminid, PA-0824
- Diamines: SQ-109

Table 1: Recommended dosage for initial treatment of tuberculosis in adults: [46]

DOSAGE		
Drug	**Daily dose**	**Thrice weekly dose**
Isoniazid	5mg/kg, max 300 mg	10mg/kg, max 900mg
Rifampicin	10mg/kg, max 600 mg	10mg/kg, max 600mg
Pyrazinamide	25mg/kg, max 2g	35mg/kg, max 3g
Ethambutol	15mg/kg	30mg/kg

Length of treatment for extrapulmonary tuberculosis:

First line regimen (2HRZE/4HRE) is given for a period of 6 months in peripheral lymph node tuberculosis and abdominal tuberculosis. The same first line regimen is recommended for a longer period of 9 months and 12 months in adults and children respectively, in case of TB meningitis. [52, 56, 57] In ocular tuberculosis HRZE/4HRE is given for a period of 6-9 months. Corticosteroids can be used as an adjunctive therapy in ocular tuberculosis. [50,51] In TB meningitis, an intensive phase of 2 months of HRZE and a continuation phase of at least 7 months of HRE is recommended for both adult and children. [56, 57] In case of visual impairment Streptomycin is used instead of Ethambutol. In central nervous system tuberculoma, Anti-Tubercular Treatment (ATT) is continued for 9-12 months and neuroimaging is done at 3 months. In case of paradoxical increase in size or number of tuberculoma, it requires corticosteroid treatment along with ATT. [54] ENT tuberculosis is treated with 2HRZE/4-7 HRE and steroids should be avoided.

Pleural tuberculosis is supported by ADA (Adenine Deaminase) level measurement. If the ADA level is >70U/L, then it is highly likely to be pleural tuberculosis. If the level is <40 U/L, it is less likely to be pleural tuberculosis. 2HRZE/4HRE is given for a period of 6 months in pleural tuberculosis. ADA level of >39 IU/ml in ascitic fluid is suggestive of abdominal tuberculosis. Abdominal tuberculosis is treated with the first line regimen of 2HRZE/4HRE for 6 months. Urogenital tuberculosis in adults and children are treated with the same first line regimen for 6 months.[50,51] For tuberculosis of spine, the treatment recommended is 2HRZE/10HRE for a period of 12months and it can be extended to 18 months.[58] Cutaneous tuberculosis is treated with 2HRZE/4HRE both in adult and children.

Treatment in special groups: [50, 51]

Pregnant and breast feeding women:

Drugs such as streptomycin, ethionamide, prothionamide and quinolone should be avoided in pregnant and breast feeding women. They are treated with HRZE and 10 mg of pyridoxine. There is no need to cease breast feeding.

Women who need contraception:

Hormonal contraception should be avoided when treated with rifampicin and month after the treatment.

Patients with kidney impairment:

They need dose adjustment of the ATT drugs used.

Liver disease:

Liver disease patients do not require any change in the first line treatment. Regimen containing no hepatotoxic drugs such as 18-24 months of Streptomycin, Ethambutol and Fluoroquinolone can be used in these patients.

Recommendation of steroids in extra pulmonary tuberculosis:

Steroids are recommended in HIV negative individuals and in HIV positive individuals with no opportunistic infections in case of TB meningitis [55] and TB pericarditis with pericardial effusion [53]. Steroids are given for a period of 4 weeks and then tapered. Gastro intestinal bleeding, increase in sugar level, increase in blood pressure and bacterial infections are the adverse effects of corticosteroid. [50, 51]

Medicines recommended for treatment of Rifampicin resistance tuberculosis and Multi Drug Resistant -Tuberculosis: [66]

Group A: Fluoroquinolones

- Levofloxacin
- Moxifloxacin
- Gatifloxacin

Group B: Second line injectable agents

- Amikacin
- Capreomycin
- Kanamycin
- (Streptomycin)

Group C: Other core second line agents

- Ethionamide/ prothionamide
- Cycloserine/ terizidone
- Linezolid
- Clofazimine

Group D: Add on agents

D1	Pyrazinamide Ethambutol High dose Isoniazid
D2	Bedaquiline Delamanide
D3	p- aminosalicylic acid Imipenem- Cilastatin Meropenem Amoxicillin- clavulanate (Thioacetazone)

Materials and methods

MATERIALS AND METHODS:

STUDY POPULATION:

Patients with extra pulmonary tuberculosis

STUDY LOCALE:

Diagnostic Microbiology Laboratory, PSG Hospitals, Coimbatore.

STUDY PERIOD:

One year (September 2017 – September 2018)

SAMPLE SIZE ESTIMATION:

$n= 4pq/d^2$

Where n = required sample size

P = expected prevalence

q = 100-p

d = degree of prevalence

SAMPLE SIZE:

500 extra pulmonary samples

SAMPLING METHOD:

Convenient sampling

INCLUSION CRITERIA:

All extra pulmonary samples (cerebrospinal fluid, pus, urine, lymph node, tissue biopsies, pleural fluid and other body fluids) from patients who are clinically suspected of extra pulmonary tuberculosis were included in the study.

EXCLUSION CRITERIA:

- Sputum samples
- Bronchoalveolar lavage
- Nasopharyngeal aspirate
- Tracheal aspirate

TYPE OF STUDY:

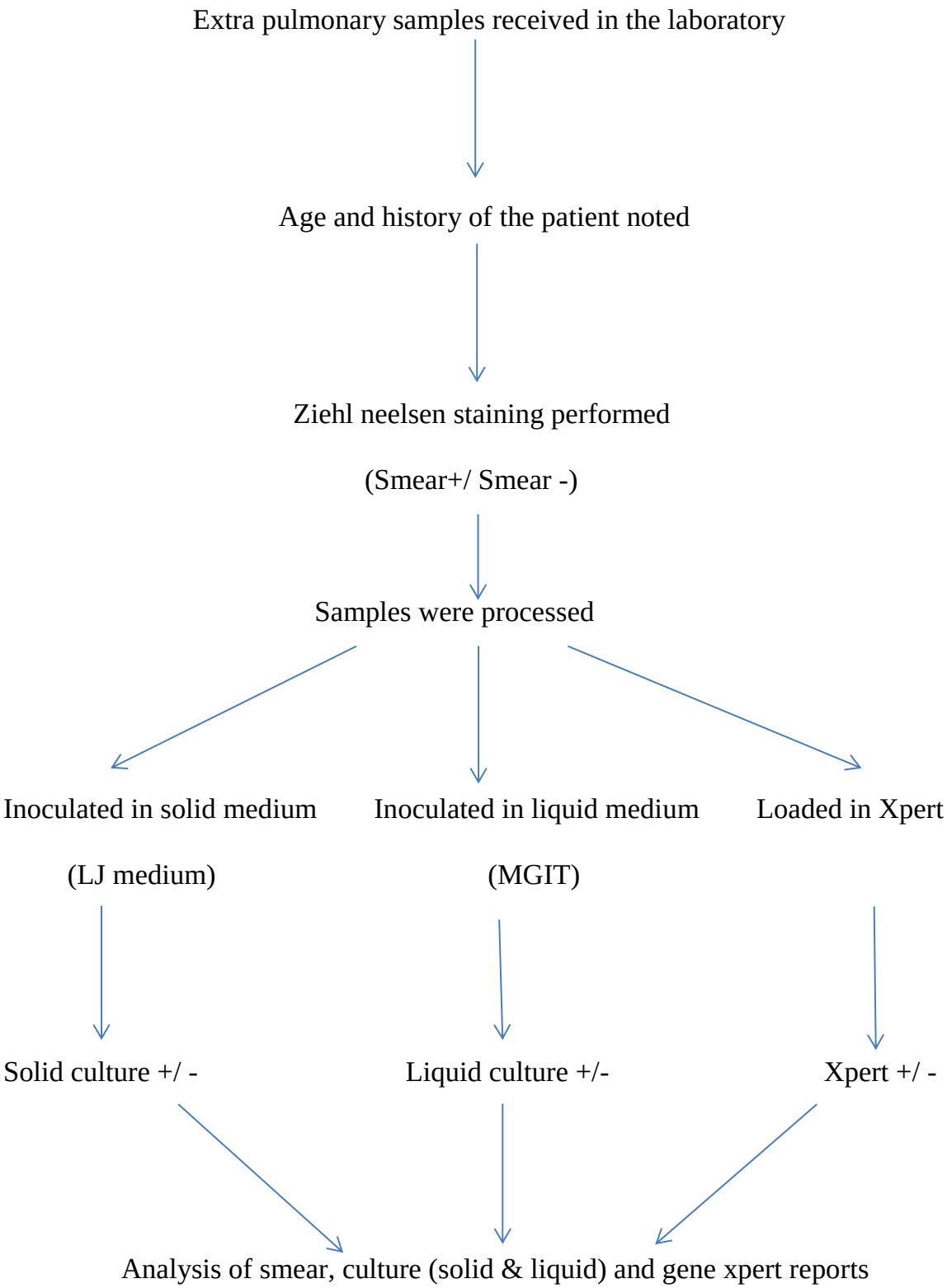
Prospective study

ETHICAL CLEARANCE:

This study is undertaken in the Diagnostic Microbiology Laboratory Department of Microbiology, PSG Hospitals, Institutional Human Ethical Clearance was obtained, proof of which has been attached.

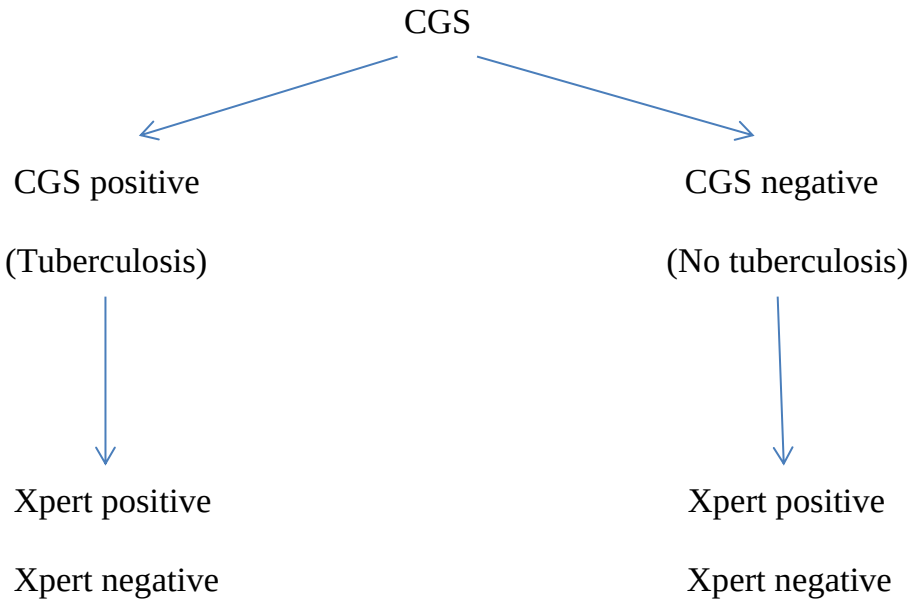
METHODOLOGY:

FLOW CHART:



Flow chart showing samples analysed against Composite Gold Standard (CGS):

500 extrapulmonary samples for pooled analysis against Composite Gold Standard



All extra pulmonary samples that were sent to Diagnostic Microbiology Laboratory were included in the study. Around 500 extra pulmonary samples were processed. Initially for all the extra pulmonary samples, smears were made, stained with Ziehl Neelsen stain and looked for the presence or absence of *Mycobacterium tuberculosis* (acid fast bacilli). Then solid and liquid cultures were performed for all samples. The composite gold standard were fixed which included clinical features, solid or liquid culture, radiology and histology results obtained from medical records. Patient is considered as tuberculosis if any one of the composite gold standard showed positive result. All samples were simultaneously loaded in the Gene Xpert and the results of Xpert were compared with solid, liquid culture and composite gold standard. Statistical analysis was done using SPSS Software which included sensitivity, specificity, positive and negative predictive value.

SAMPLE COLLECTION:

Extrapulmonary samples received in the laboratory were initially subjected to smear microscopy after staining with ZN stain.

SMEAR PREPARATION:

CSF, ASCITIC, JOINT, PLEURAL FLUIDS:

Specimens were centrifuged at 3500 rpm for 30 minutes and the deposits were used to make smears.

Cerebrospinal fluids:

- On a clean glass slide, 2 parallel marks of about 10 mm long and 2 mm apart were made. A loopful of the sediment is then spread between these marks and the smear is allowed to dry. Another loopful is then spread over the first and allowed to dry. The process is repeated atleast 3 times. Then the smears are stained and examined.

Gastric washings:

- The specimen is centrifuged at 3500rpm for 30 minutes and the deposit was used for making smears.

Pus:

- Swabs and wound aspirates were transferred to 50 ml conical centrifuge tube containing 5-10 ml sterile distilled water, vortexed and allowed to stand for 20 minutes. Then the swabs were removed and discarded. Prior to smear and culture, the sample is decontaminated and concentrated.

Tissue:

- Specimens were finely minced using a sterile scalpel and homogenized in a sterile tissue grinder with sterile saline.

Urine:

- Entire specimen is centrifuged for 30 minutes at 3500 rpm and two smears were prepared using 10µl loop from the concentrated specimen (deposit). Another loopful is then spread over the first and allowed to dry. The process is repeated atleast 3 times. Then the smears were stained by Ziehl Neelsen using 25% sulphuric acid (for one smear) and 3% hydrochloric acid alcohol mixture (for second smear) as decolourisers. [84]

ZIEHL NEELSEN STAINING PROCEDURE:

- The slides were arranged on the levelled staining bridge with smear side up.
- Carbol fuchsin stain was applied to cover the entire surface of the slide
- The slides were heated until steam rises from the stain.
- The slides were left for 10 minutes
- Then the slides were rinsed with a gentle stream of water. Excess water is drained by tilting the slide.
- 25% sulphuric acid or 3% hydrochloric acid was used as decolouriser. The slides were covered with the decolouriser and left for 3 minutes. If carbol fuchsin stain was found to be retained, again decolourisation step was repeated.
- The slides were washed with a gentle stream of water. Excess water was drained.
- The smears were counterstained with methylene blue for 1 minute.

- Again the slides were rinsed with gentle stream of water and the slides were allowed to air dry.
- The slides were then examined under oil immersion microscopy (100x) for the presence of acid fast bacilli.

INTERPRETATION:

Smear positive: Acid fast bacilli seen (pink bacilli seen against a blue background)

Smear negative: Acid fast bacilli not seen

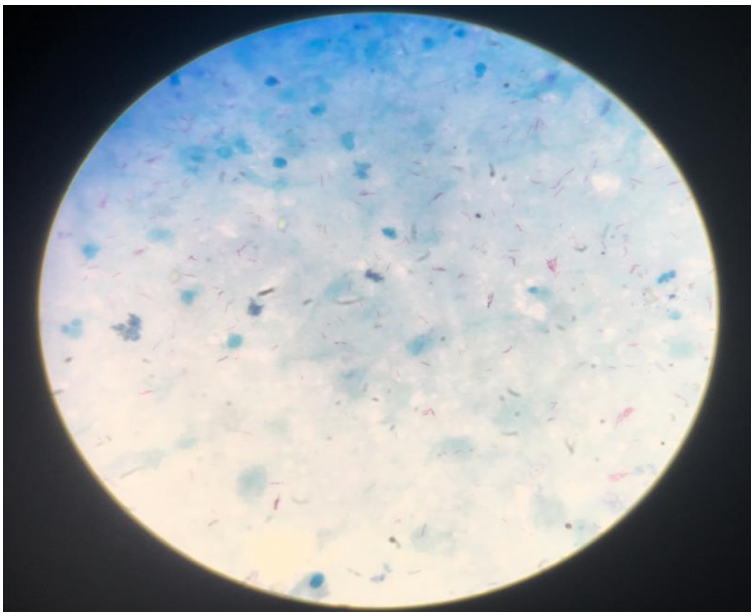


Figure 1: Acid fast staining of *Mycobacterium tuberculosis*

PROCESSING OF SPECIMENS:

The samples were processed using Modified Petroffs method.

MODIFIED PETROFF METHOD:

PROCEDURE:

The working solution of the decontamination mixture contains 2% NaOH, 1% NALC and 1.45% sodium citrate. To x ml of the sample equal volume of

decontamination mixture was added in a screw capped centrifuge tube and vortexed. And then the centrifuge tubes were allowed to stand for 15 minutes with occasional shaking at room temperature. Then centrifuged at 3000 x g for 15 minutes and the supernatant were poured off. The sediment was re suspended in 15 ml of sterile distilled water and again centrifuged at 3000 x g for 15 minutes. The supernatant was decanted and inoculated onto culture medium.

BACTEC MGIT 960 (Becton Dickinson, Sparks, MD, USA): [83]

Non radiometric automated isolation system

REAGENTS PROVIDED:

110 µl of fluorescent indicator

7 ml of broth

OADC Enrichment:

Bovine albumin, Dextrose, Polyxyethylene Stearate (POES), Catalase and Oleic acid.

MGIT PANTA:

(Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin)

PROCEDURE:

The lyophilized vial of BD BBL MGIT PANTA antibiotic mixture was mixed with BD BACTEC MGIT growth supplement. The MGIT tube was labelled with specimen number. 0.8 ml of growth supplement/ antibiotic mixture was added aseptically into the MGIT tube. Then 0.5 ml of the concentrated specimen was added to the tube. Tubes were recapped and mixed well. Then the tubes were entered in the instrument and tested automatically for about 42 days. The bottom of the tube contains an oxygen sensor ruthenium chloride pentahydrate, which fluoresces following

reduction in oxygen dissolved in the broth. Aerobically metabolizing bacteria present in the medium consumes the oxygen and thus allows the fluorescence to be detected. In case of positive growth, it was sub cultured and acid fast smear was prepared.

EXAMINATION OF MGIT TUBES:

Once the process is complete, the MGIT tubes are removed from the machine. MGIT tubes are examined for turbidity (breadcrumbs at the bottom of the tube). The number of days and hours taken to reach positivity is noted. For positive tubes (turbid tubes), 10 µl of the liquid from the MGIT tube is inoculated on to the blood agar plate and incubated at 37^0c for 48 hours. The blood agar plate is checked for growth or contamination daily. ZN smear microscopy is performed on positive MGIT tubes using direct sediment present at the bottom of tubes or after concentrating the sample to look for acid fast bacilli.

Figure2: MGIT-960 (Becton Dickinson, Sparks, MD, USA)

Figure3: BACTEC MGIT 960 showing positive growth of *Mycobacterium tuberculosis*

INNOCULATION ONTO LJ MEDIUM: [82]

After decontamination with Modified Petroff method, the pellets were reconstituted with phosphate buffer. 0.5 ml is added to the LJ medium.

INOCULATION PROCEDURES:

Before inoculation condensed moisture were removed from the medium. Disposable loops are used for culture inoculation. 0.2 -0.4 ml of the centrifuged sediment were distributed over the surface of each LJ slope. Two slopes of LJ medium were inoculated per specimen. For even distribution of the inoculum, the inoculated media were preferably incubated in slanted position for atleast 24 hours. Tops of the LJ medium were tightened mainly to minimize the evaporation and drying of the medium.

INCUBATION OF CULTURES:

All cultures should be incubated at 35- 37 ^{0} c until growth is observed or discarded as negative after 8 weeks.

CULTURE EXAMINATION:

EXAMINATION SCHEDULE:

LJ slopes are examined for contamination at 48-72 hours. Grossly contaminated ones are discarded. The cultures are then examined weekly up to 8 weeks for the presence of mycobacterial growth. In case if the LJ slope did not show any growth at 8 weeks, but the smear microscopy is positive for acid fast bacilli, then the slopes are incubated further for 12 weeks.

Figure 4: Un inoculated Lowenstein Jensen medium

Figure 5: LJ medium showing growth of *Mycobacterium tuberculosis*

GENE XPERT MTB/RIF (Cepheid, Sunnyvale, CA, USA):

REQUIREMENTS:

- Biosafety cabin
- Gene xpert system
- Xpert MTB/RIF cartridge
- Sample reagent
- Sterile disposable transfer pipette
- Sterile screw capped container
- Discard containers
- Permanent marker pen

GENE XPERT PROCEDURE: [84]

MATERIALS AND REAGENTS PROVIDED:

Bead 1: primer, probes, KCL, MgCl2, HEPES (4-(2- hydroxyethyl)- 1- piperazine ethane sulfonic acid), Bovine serum albumin (BSA), pH: 8.

Bead 2: primer, probe, KCL, MgCl2, dNTP (deoxyriboucleotide triphosphate), HEPES, BSA, pH 7.2.

Bead 3: approximately 6000 non-infectious sample preparation control spores.

Reagent 1: tris buffer, EDTA an surfactants

Reagent 2: tris buffer, EDTA an surfactants

Sample reagent: sodium hydroxide and isopropanol.

PROCEDURE:

To the specimen, double volume of buffer solution was added in a centrifuge tube, vortexed and then left at room temperature for 15 minutes. Then 3 ml of the vortexed mixture was added to the sample loading area of the cartridge without air bubbles. The lid of the cartridge was closed. Then the barcode on the gene xpert cartridge was scanned, patient ID entered and the instrument module was opened and the cartridge was loaded. The instrument module door was closed. Once the test gets over, the door lock will be released.

INTERPRETATION OF GENE XPERT RESULTS: [85]

The results from Gene Xpert assay indicate whether *Mycobacterium tuberculosis* is detected in the sample or not. When the result comes as invalid, the test should be repeated. The result also says whether rifampicin resistance was detected or not detected.

- **MTB detected**: target DNA is detected and depending on the cycle threshold value, the result will be displayed as high, medium, low or very low detection.
- Concentration of bacilli in the sample is defined by the cycle threshold range.

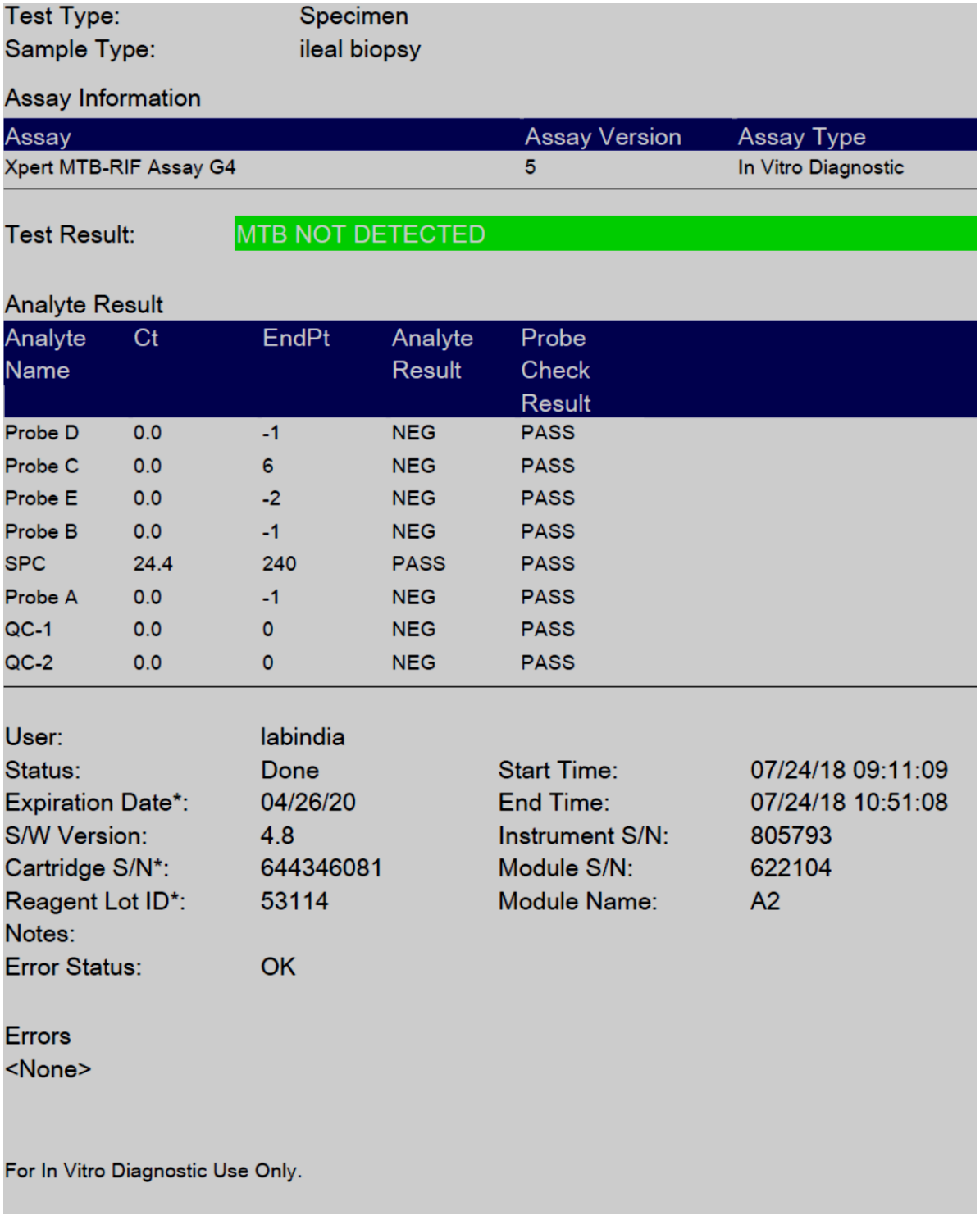
Table 2: Concentration of bacilli in the sample and the cycle threshold range

Concentration of bacilli	**Cycle threshold range (C_T range)**
High	<16
Medium	16-22
Low	22-28
Very low	>28

- **MTB detected, RIF resistance detected**: MTB target is present within the sample and mutation in rpo B gene detected.
- **MTB detected, RIF resistance not detected**: MTB target is present within the sample and no mutation in rpo B gene detected.
- **MTB detected, RIF resistance indeterminate**: MTB target is present within the sample and RIF resistance could not be determined due to insufficient signal detection.
- **MTB not detected**: MTB target is not detected within the sample.
- **Invalid**: MTB DNA cannot be determined. Repeat the test in this case.
- **Error**: MTB cannot be determined. Sample processing control shows no result and probe check results failed. The test is repeated.
- **No result**: MTB cannot be determined. The test is repeated.

Figure 6: Gene Xpert MTB/RIF (Cepheid, Sunnyvale, CA)

Figure 7: Gene Xpert MTB/RIF cartridge



Test Type: Specimen
Sample Type: ileal biopsy

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-RIF Assay G4	5	In Vitro Diagnostic

Test Result: MTB NOT DETECTED

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
Probe D	0.0	-1	NEG	PASS
Probe C	0.0	6	NEG	PASS
Probe E	0.0	-2	NEG	PASS
Probe B	0.0	-1	NEG	PASS
SPC	24.4	240	PASS	PASS
Probe A	0.0	-1	NEG	PASS
QC-1	0.0	0	NEG	PASS
QC-2	0.0	0	NEG	PASS

User:	labindia		
Status:	Done	Start Time:	07/24/18 09:11:09
Expiration Date*:	04/26/20	End Time:	07/24/18 10:51:08
S/W Version:	4.8	Instrument S/N:	805793
Cartridge S/N*:	644346081	Module S/N:	622104
Reagent Lot ID*:	53114	Module Name:	A2
Notes:			
Error Status:	OK		

Errors
<None>

For In Vitro Diagnostic Use Only.

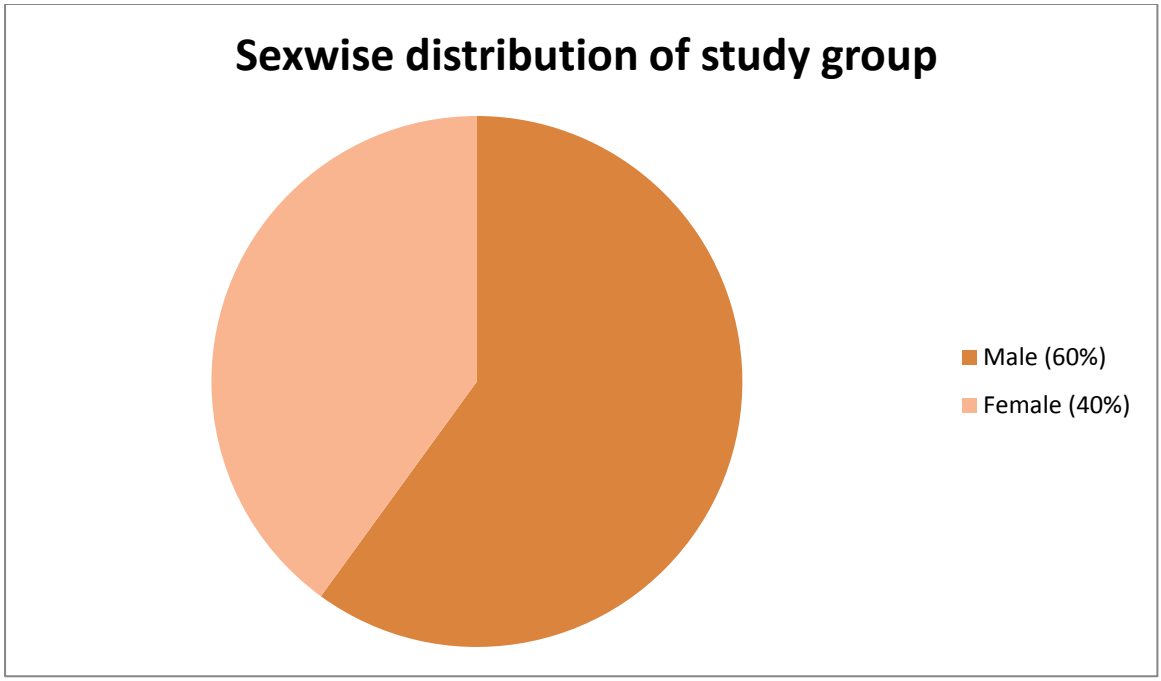
Figure 8: Printable report of Gene Xpert MTB/RIF

Results and Analysis

RESULTS

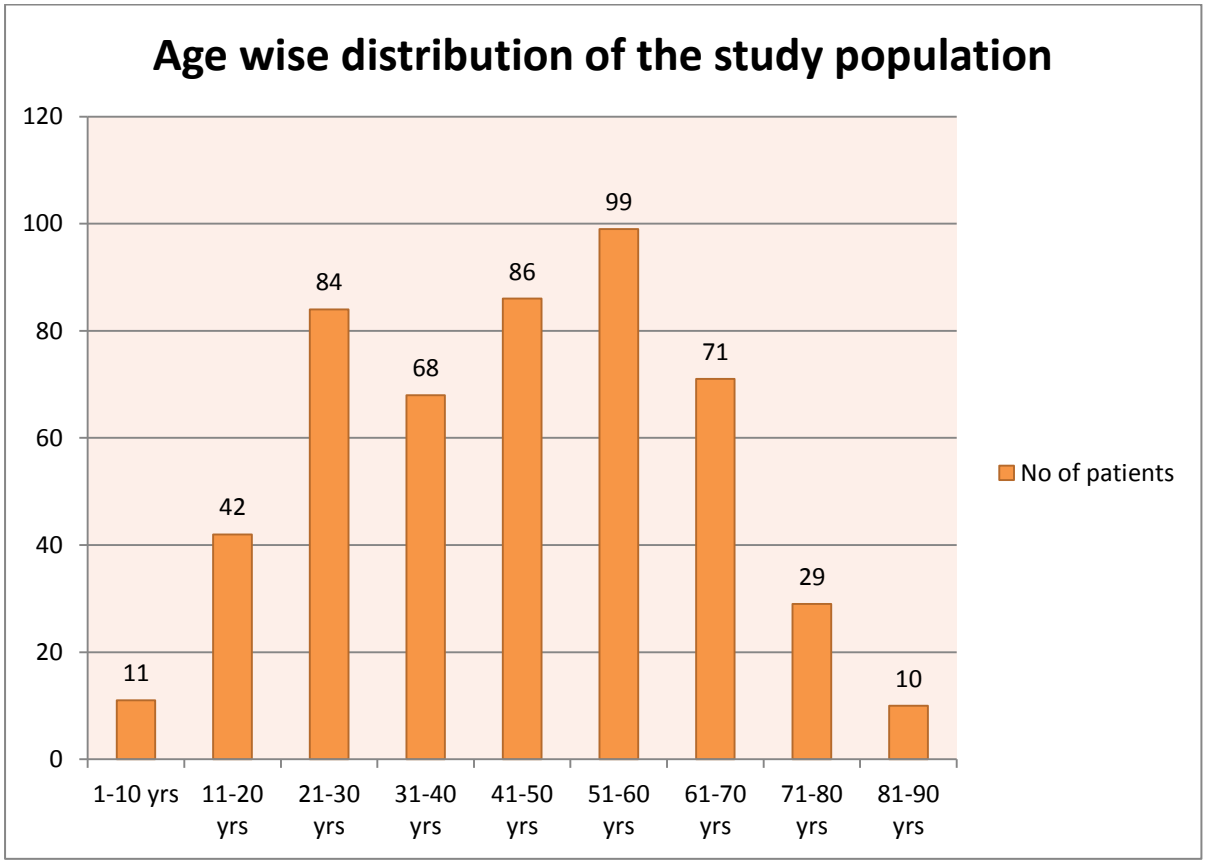
All extra pulmonary samples that were received in the laboratory were processed. Among the 500 extra pulmonary samples, male patients (60%) and the remaining 40% were obtained from female patients.

Figure 9: Sex wise distribution of study group



The study population were analysed based on their age. Maximum number of patients fall under the age group 51-60 years (99 patients i.e. 19.8%) followed by 86 patients between 41-50 years (17.2%) and 84 patients between 21-30 years (16.8%). The least incidence was seen under extremes of age, i.e. age group less than 10 years (2.2%) and between 81-90 years (0.2%) as shown in figure 10. 20 patients (4%) among these 500 patients were HIV positive. The mean age of the patient was 45.08 years.

Figure 10: Age wise distribution of the study group



Among the 500 extra pulmonary samples that were received in the laboratory, maximum numbers of samples were from CSF, around 167 samples (33.4%). 109 samples (21.8%) were from pleural fluid**,** 71 samples (14.2%) were obtained from tissue specimens. Among the 71 tissue samples, 68 samples were from ileal tissue, 2 samples were from endometrial tissue and 1 sample from caecal biopsy. 10.2% of samples were pus samples, 9.2% of samples were from lymph node and 8% of fluid samples included ascitic fluid (28 samples), pericardial fluid (4 samples) and gastric fluid (8 samples). Least number of sample obtained were from bone marrow (1.4%) and urine (1.8%) as shown in table 3.

Table 3: Extra pulmonary samples analysed in the study.

TYPE OF SAMPLE	NO. OF SAMPLES	PERCENTAGE
CSF	167	33.4%
Pleural fluid	109	21.8%
Tissue	71	14.2%
Pus	51	10.2%
Lymph node	46	9.2%
Other fluids (pericardial, gastric, ascitic fluids)	40	8%
Urine	9	1.8%
Bone marrow	7	1.4%
Total	**500**	**100%**

Smears were made for all 500 extrapulmonary samples and stained with Ziehl Neelsen stain. After staining all the smears were examined for acid fast bacilli which appear bright red against a blue background. And all these samples were inoculated onto Lownstein Jensen media (solid media) and Mycobacterial growth indicator tube (MGIT 960-liquid media). Simultaneously they were loaded in Gene Xpert MTB/RIF to detect *Mycobacterium tuberculosis* and rifampicin resistance.

Table 4: Number of extra pulmonary samples that are detected by smear microscopy, culture and Xpert MTB/RIF

TYPE OF SAMPLE	SMEAR MICROSCOPY POSITIVE	LJ CULTURE POSITIVE	MGIT POSITIVE	XPERT MTB/RIF POSITIVE
CSF (n=167)	0	6	6	6
Pleural fluid (n=109)	4	10	10	10
Tissue (n=71)	1	8	8	8
Lymph node (n=46)	3	12	13	12
Pus (n=51)	12	19	19	19
Urine (n=9)	0	1	1	1
Bone marrow (n=7)	1	0	0	0
TOTAL	**21**	**56**	**57**	**56**

Among the 500 extrapulmonary samples, 21 smears were positive for acid fast bacilli. 56 samples were positive by solid culture (LJ culture) and 57 samples were positive for *Mycobacterium tuberculosis* in liquid culture (MGIT) and 56 samples were positive by Gene Xpert MTB/RIF. Rifampicin resistance was detected in 5.35% (3 out of 56).

Figure 11: Sex distribution of positive samples

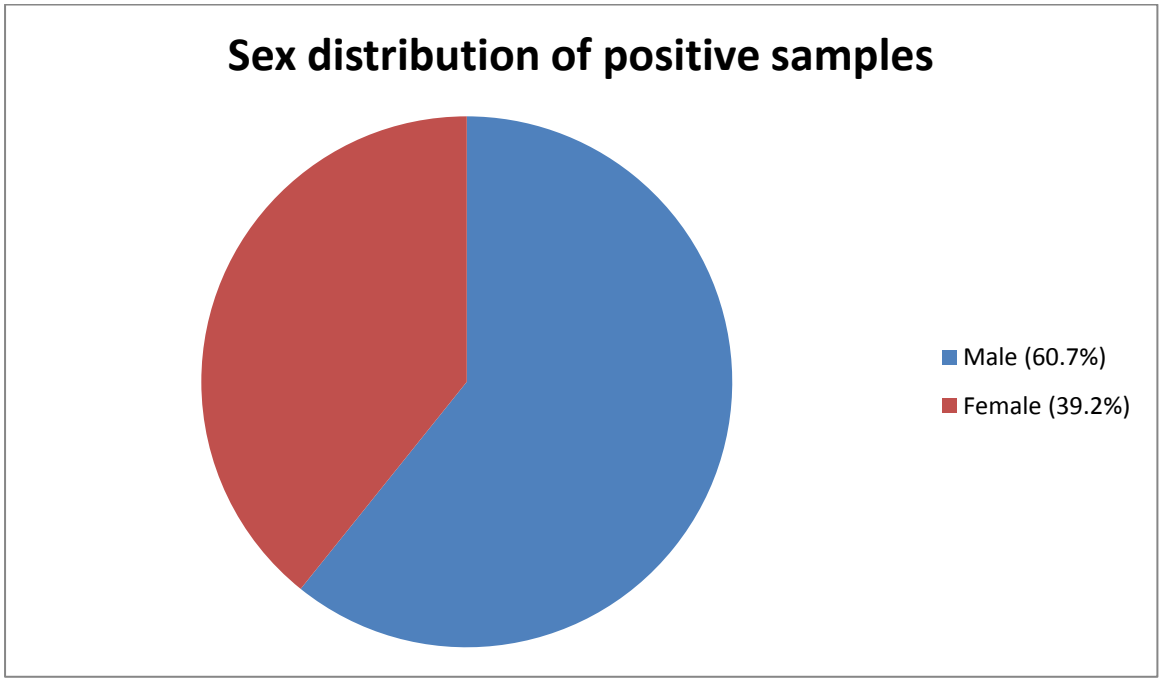


Figure 11 shows the sex distribution of positive samples detected by Xpert MTB/RIF.

Around 60.7% males and 39.2% females were positive by Xpert MTB/RIF.

Figure 12: Age wise distribution of positive samples

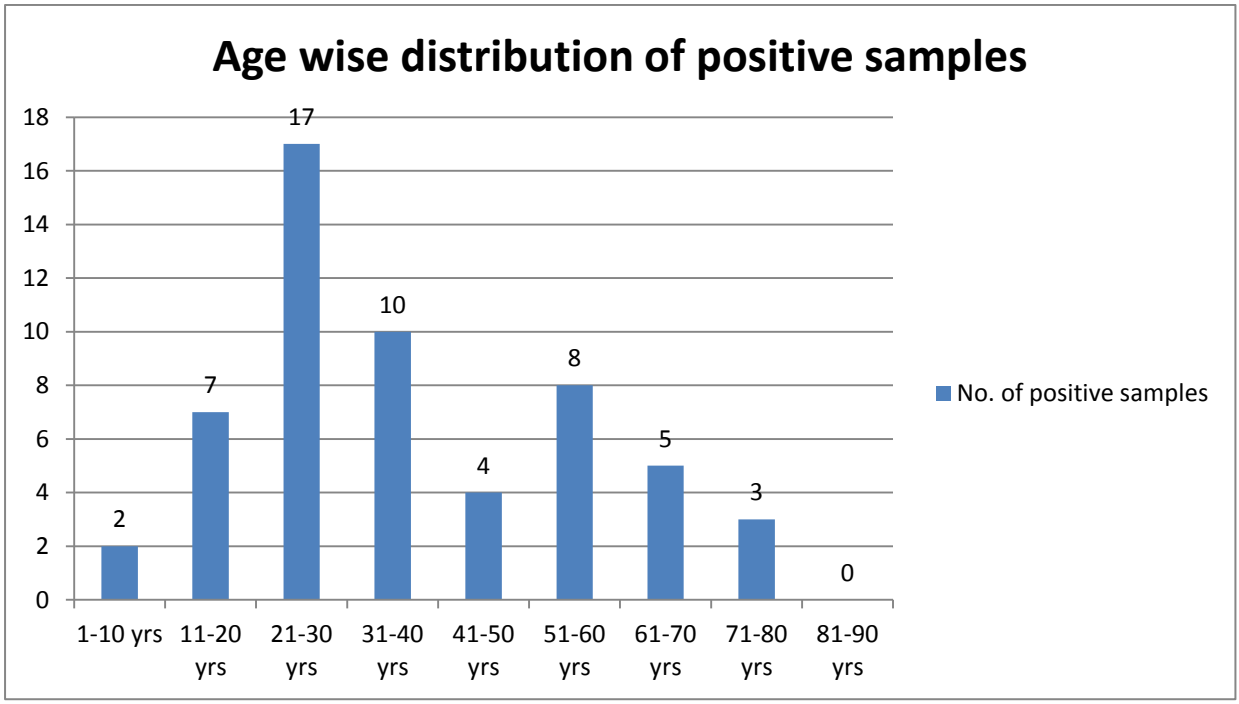


Figure 12 shows age wise distribution of positive samples by Xpert MTB/RIF. Among the 56 samples detected by Xpert MTB/RIF, maximum number of positive sample was between 21-30 years (30.4%), followed by 31-40 years (17.9%). Least number of positivity is seen between 1-10 years and 71-80 years. None of the samples were positive between 81-90 years of age.

Table 5: Sample wise distribution of positive cases

SAMPLES	**NO. OF SAMPLES POSITIVE BY XPERT MTB/RIF**
CSF (n= 167)	6
Pleural fluid (n=109)	10
Tissue (n=71)	8
Lymph node (n=46)	12
Pus (n=51)	19
Urine (n=9)	1
TOTAL	**56**

Among the 56 samples that were detected positive for *Mycobacterium tuberculosis* by Xpert MTB/RIF, 19 pus samples showed positive for *Mycobacterium tuberculosis*, followed by lymph node (12) and pleural fluid (10). Only 6 samples were detected positive for *Mycobacterium tuberculosis* in cerebrospinal fluid. Only 1 sample was positive in urine as shown in table 5.

Figure 13: Flow chart showing the results of solid and liquid culture and Gene Xpert MTB/RIF in smear positive and smear negative cases.

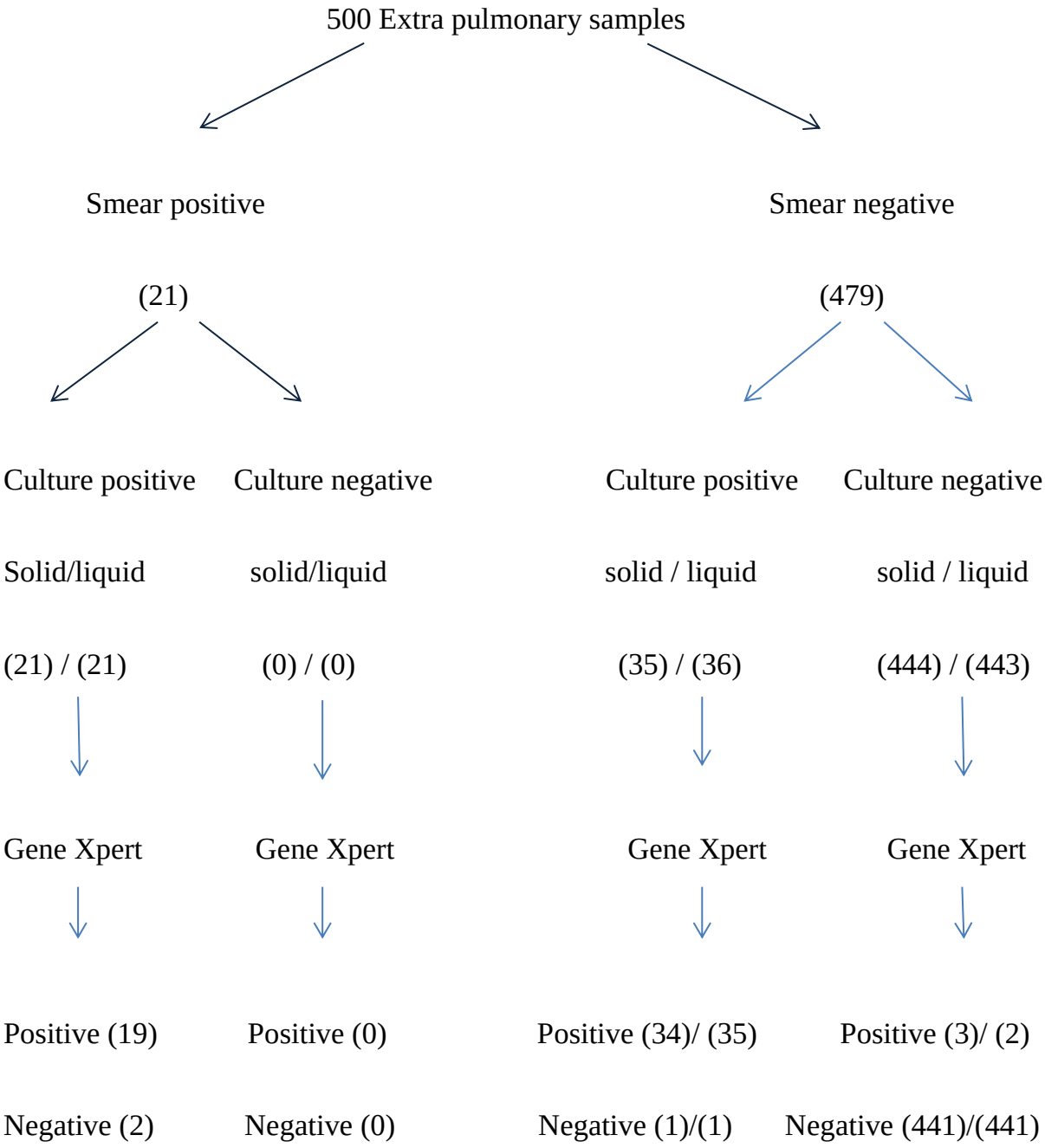
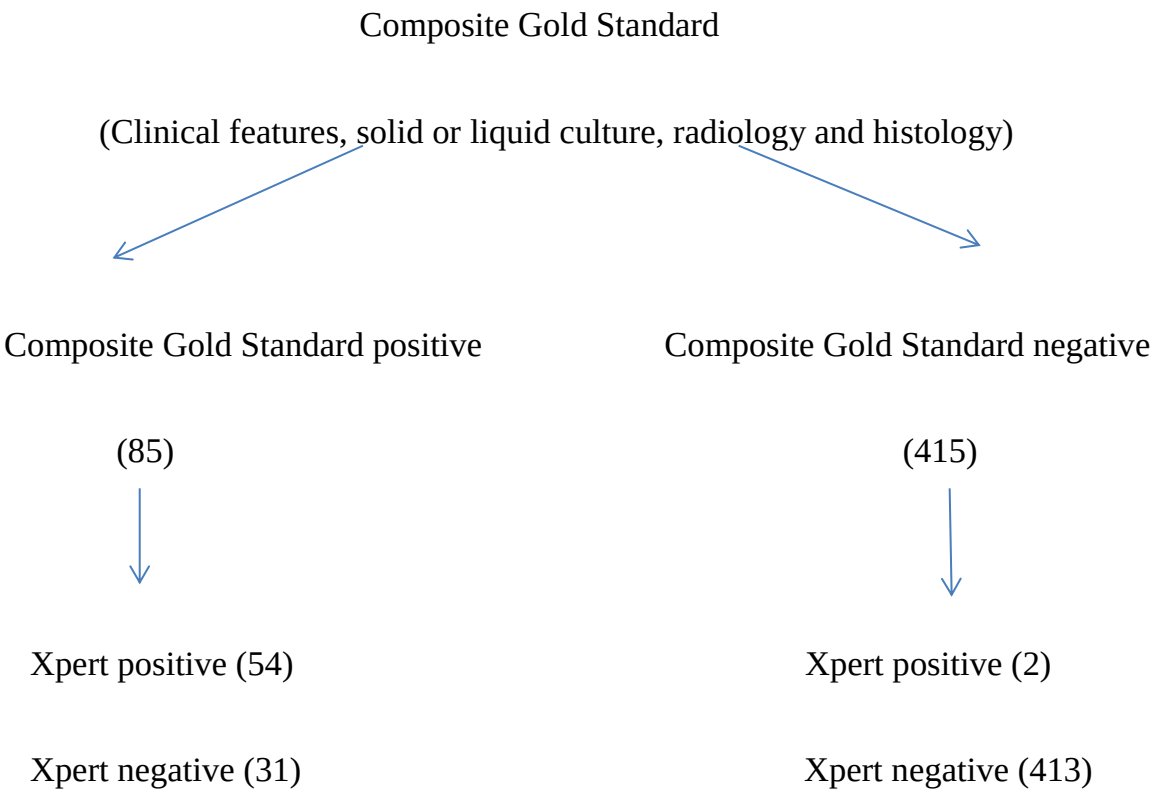


Figure 14: Flow chart showing the results of Composite gold standard and Xpert MTB/RIF assay



Out of 500 samples, 20 samples were from HIV positive patients. Radiology detected tuberculosis in 18 samples out of 56 samples that were detected positive by Xpert MTB/RIF. Histopathology detected Mycobacterium tuberculosis in 30 samples out of 500 samples. Among these 30 samples only 14 samples were detected positive both by histopathology and Xpert MTB/RIF.

Table 6: Sample wise distribution of positive cases by radiology, histopathology and clinical features

TYPE OF SAMPLE	DECTECTED POSITIVE BY RADIOLOGY	DETECTED POSITIVE BY HISTOPATHOLOGY	CLINICAL FEATURES
CSF (n=167)	3	2	6
Pleural fluid (n=109)	1	8	5
Tissue (n=71)	4	2	8
Pus (n=51)	4	4	0
Lymph node (n=46)	4	11	6
Other fluids (n=40)	1	2	2
Bone marrow (n=7)	1	1	0
TOTAL	**18**	**30**	**27**

Table 7: Comparative evaluation of Xpert MTB/RIF with smear.

XPERT	**SMEAR**		**TOTAL**
	POSITIVE	**NEGATIVE**	
POSITIVE	19	37	56
NEGATIVE	2	442	444
TOTAL	21	479	500

Pooled Sensitivity of Xpert MTB/RIF = 90.5% (95% CI: 0.68-0.98)

Pooled Specificity of Xpert MTB/RIF = 92.3% (95% CI: 0.89-0.94)

Positive predictive value = 33.9%

Negative predictive value = 99.5%

From table 7, the Xpert MTB/RIF showed a sensitivity of 90.5% and a specificity of 92.3%. The positive and negative predictive value was around 33.9% and 99.5% respectively. 19 samples showed positive by smear microscopy and Xpert MTB/RIF. 442 samples were negative for acid fast bacilli in smear microscopy and *Mycobacterium tuberculosis* was not detected in 442 samples by Xpert MTB/RIF. Out of 56 samples that were detected for *Mycobacterium tuberculosis*, 37 showed negative for acid fast bacilli in smear microscopy by Ziehl Neelsen staining. Among 444 samples that were negative for *Mycobacterium tuberculosis* by Xpert MTB/RIF, 2 samples were detected positive for acid fast bacilli in smear microscopy.

Table 8: Comparative evaluation of Xpert MTB/RIF with solid culture (LJ)

XPERT	SOLID CULTURE(LJ)		TOTAL
	POSITIVE	NEGATIVE	
POSITIVE	53	3	56
NEGATIVE	3	441	444
TOTAL	56	444	500

Pooled Sensitivity of Xpert MTB/RIF = 94.6% (95% CI: 0.84-0.98)

Pooled Specificity of Xpert MTB/RIF = 99.3% (95% CI: 0.97-0.99)

Positive predictive value = 94.6%

Negative predictive value = 99.3%

From table 8, Xpert MTB/RIF when compared with solid culture showed a sensitivity of 94.6% and a specificity of 99.3%. The positive and negative predictive values were 94.6% and 99.3% respectively. Around 53 samples were detected positive for *Mycobacterium tuberculosis* in both culture and Xpert MTB/RIF. 441 samples were negative by culture and Xpert MTB/RIF. Among the 56 samples that were detected by Xpert MTB/RIF, 3 samples did not grow *Mycobacterium tuberculosis* in culture. Out of 444 samples 3 were positive by culture for *Mycobacterium tuberculosis*, which were not detected in Xpert MTB/RIF.

Table 9: Comparative evaluation of Xpert MTB/RIF with liquid culture

XPERT	LIQUID CULTURE(MGIT)		TOTAL
	POSITIVE	NEGATIVE	
POSITIVE	54	2	56
NEGATIVE	3	441	444
TOTAL	57	443	500

Pooled Sensitivity of Xpert MTB/RIF = 94.7% (95% CI: 0.84-0.98)

Pooled Specificity of Xpert MTB/RIF = 99.5% (95% CI: 0.98-0.99)

Positive predictive value = 96.4%

Negative predictive value = 99.3%

From table 9, Xpert MTB/RIF when compared with solid culture showed a sensitivity of 94.7% and a specificity of 99.5%. The positive and negative predictive values were 96.4% and 99.3% respectively. Around 54 samples were detected positive for *Mycobacterium tuberculosis* in both culture and Xpert MTB/RIF. 441 samples were negative by culture and Xpert MTB/RIF. Among the 56 samples that were detected by Xpert MTB/RIF, 2 samples did not grow *Mycobacterium tuberculosis* in culture. Out of 444 samples 3 were positive by culture for *Mycobacterium tuberculosis*, which were not detected in Xpert MTB/RIF.

Table 10: Comparative evaluation of Xpert MTB/RIF with Composite Gold Standard

XPERT	CGS		TOTAL
	POSITIVE	NEGATIVE	
POSITIVE	54	2	56
NEGATIVE	31	413	444
TOTAL	85	415	500

Pooled Sensitivity of Xpert MTB/RIF = 63.5% (95% CI: 0.52- 0.73)

Pooled Specificity of Xpert MTB/RIF = 99.5% (95% CI: 0.98-0.99)

Positive predictive value = 96.4%

Negative predictive value = 93%

From table 10, the sensitivity and specificity of Xpert MTB/RIF when compared with CGS were 63.5% and 99.5%. 96.4% and 935 were the positive and negative predictive value of Xpert MTB/RIF when compared with CGS.

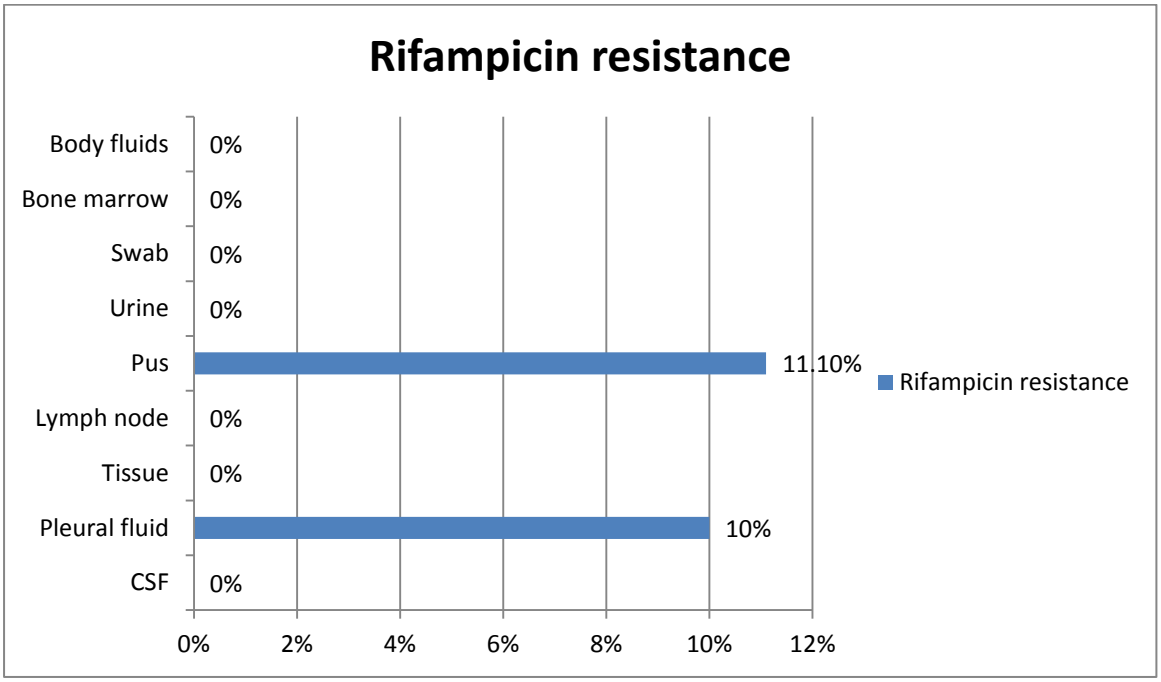
Table 11: Results of Xpert MTB/RIF compared to liquid culture among various Samples

SAMPLES	SENSITIVITY (100%)	SPECIFICITY (100%)
CSF	100	100
PLEURAL FLUID	100	100
TISSUE	100	100
LYMPH NODE	92.3	100
PUS	100	100
URINE	100	100
BONE MARROW	Not calculable	100

The sensitivity and specificity of CSF, pleural fluid, tissue specimen, pus and urine were 100% when compared with the gold standard culture technique. But in case of lymph node the sensitivity and specificity of Xpert MTB/RIF when compared with culture were 92.3% and 100% respectively. In case of bone marrow samples, among the 7 total samples received in the laboratory, none of the sample showed positive for *Mycobacterium tuberculosis* in both liquid culture and Xpert MTB/RIF. Therefore the sensitivity of bone marrow samples cannot be calculated as shown in table 11.

RIFAMPICIN RESISTANCE:

Figure 15: Rifampicin resistance detected by Xpert MTB/RIF among extra pulmonary samples.



Rifampicin resistance were detected in 3 out of 56 extra pulmonary samples (5.35%) that were positive by Xpert MTB/RIF. Among the 3 samples, 11.1% resistance is seen in pus sample and 10% of rifampicin resistance is seen in pleural fluid.

Among the 500 extra pulmonary samples, 21 samples were positive by smear microscopy, 57 samples were positive for *Mycobacterium tuberculosis* by liquid culture, 56 samples were positive by solid culture and 56 samples were detected *Mycobacterium tuberculosis* by Xpert MTB/RIF. Rifampicin resistance was detected by Xpert MTB/RIF in only 3 extra pulmonary samples (one from pleural fluid and two from pus sample).

Discussion

DISCUSSION

Extra pulmonary tuberculosis is an important cause of mortality and morbidity in high prevalence country like India, therefore there is need for prompt diagnosis of extra pulmonary tuberculosis mainly to reduce Tuberculosis burden. Rapid diagnosis of *Mycobacterium tuberculosis* in extra pulmonary samples is essential for effective treatment and to reduce the emergence and spread of multi drug resistant tuberculosis (MDR-TB). EPTB diagnosis is difficult due to its paucibacillary nature and its non-specific clinical signs and symptoms. As ZN smear microscopy is less sensitive due to the requirement of large bacillary load and conventional culture method which requires time, trained persons and biosafety cabin, Xpert MTB/RIF assay is a rapid diagnostic tool that offers accurate results in less than 2 hours.

In our study, majority of samples were from age group 51-60 years. Higher prevalence (30.4%) of Extrapulmonary tuberculosis was noted in younger age of 21-30 years, which is similar to a study by Arora VK which showed higher prevalence among 23.4+/- 12.8 years. [81]

Around 60.7% males and 39.2% females were positive for *Mycobacterium tuberculosis* by Xpert MTB/RIF. There were more males in our study but there is no statistical significance in gender difference seen in our study.

As per Kuppuswamy socioeconomic scale [97], 51.8% and 33.9% of patients with extrapulmonary tuberculosis belonged to upper lower class (IV) and lower class (V) respectively in our study.

In the present study, out of 500 extra pulmonary samples, 21 samples (4.2%) were Acid fast bacilli positive by ZN smear microscopy. A total of 56 samples (11.2%) and 57 samples (11.4%) were positive for *Mycobacterium tuberculosis* by solid and liquid culture respectively. only 56 samples (11.2%) were positive by Xpert MTB/RIF.

The Gene Xpert MTB/RIF assay (Cepheid USA) cartridge based nucleic acid amplification test is a newly developed, WHO recommended, automated diagnostic molecular test based on nested real time PCR and molecular beacon technology with a sensitivity of detecting 131 cfu /ml of *Mycobacterium tuberculosis* in the specimen. *Mycobacterium tuberculosis* by Xpert MTB/RIF was detected in 3.6% of CSF samples, 9.2% of pleural fluid, 11.3% of tissue samples, 37.3% of pus samples, 26.1% of lymph node samples and 11.1% of urine samples. Out of 56 positive extra pulmonary samples 3 showed rifampicin resistance. Of the 500 samples, 18 and 30 patients were detected positive for extra pulmonary tuberculosis by radiology and histopathology respectively. Only 27 patients presented with clinical features of extra pulmonary tuberculosis.

Although Gene xpert MTB/RIF has been recommended by WHO for testing extra pulmonary samples recently, validation for Gene Xpert MTB/RIF is available mostly for pulmonary tuberculosis. Mortality and morbidity due to tuberculosis is higher in low and middle income countries. Extra pulmonary tuberculosis incidence is higher in patients co-infected with HIV. In our study around 80% of HIV patients had extra pulmonary tuberculosis.

Diagnosis of extra pulmonary tuberculosis is challenging because of the lack of rapid diagnostic tools. So delay in initiation of treatment often leads to sequelae and death.As the solid culture had a contamination rate of 11% and the rate of isolation of *Mycobacterium tuberculosis* in MGIT (liquid medium) is 14.81% greater than solid culture,[92] liquid culture was considered as gold standard method in our study.

We found that the Xpert MTB/RIF had a pooled sensitivity of 94.7% and 99.5% when compared with gold standard liquid culture (MGIT 960). A study from South India showed a lower sensitivity and specificity of 89% and 74% respectively when compared to our study. [86] Another published work done in Mumbai showed lower sensitivity of 50% but higher specificity of 100% when compared to our study. [78]

Table 12: Showing sensitivity and specificity of Xpert MTB/RIF for extra-pulmonary samples in various studies.

STUDY	YEAR	SENSITIVITY	SPECIFICITY
Lawn SD et al (Italy)	2011	73.3%	99.2%
Causse et al (Spain)	2011	95%	100%
Hilleman et al (Germany)	2011	77.3%	98.2%
Ligthelm et al (South Africa)	2011	96.7%	90%
Armand et al (France)	2011	50%	100%
Vadwai et al (India)	2011	50%	100%

Nichol MP et al (South Africa)	2012	77.3%	98.2%
Tortoli et al(Italy)	2012	81%	99.8%
Lawn SD et al (South Africa)	2012	79%	97.3%
Siddiqui M et al (India)	2013	70%	100%
Held M et al (London)	2014	95.65%	96.2%
Denkinger et al	2014	83.1%	98.7%
Sharma et al (India)	2014	71%	95%
Scott et al (South Africa)	2014	47%	93%
Narute s et al (India)	2015	77.8%	84.2%
Marouane et al (Tunisia)	2015	84.3%	94.3%
Rufai et al (India)	2015	55%	100%
Iram et al (Pakistan)	2015	80%	96%
Suzana et al (India)	2016	62%	100%
UB Singh et.al (Spain)	2016	100%	99.62%
Somily et al (Saudi Arabia)	2016	100%	98.9%
PRESENT STUDY	**2018**	**94.7%**	**99.5%**

The median time taken for growth in liquid medium (MGIT 960) in our study was 15 days and the mean time for positivity in liquid medium was around 17.9 days which is consistent with studies done in Spain and Pakistan. [89,90] A study conducted in Hyderabad, India in 2013 found that the average turnaround time was 23.13 days which is higher than our present study. [60] When the Xpert MTB/RIF results for detection of *Mycobacterium tuberculosis* were medium or low, the time for positivity in liquid medium was around 15 days and for very low detection it was around 25 days. A work done in Spain showed the time for positivity in MGIT as 17 days and 20.5 days for medium/ low results and very low results in Xpert MTB/RIF assay. [89]Conventional technique of smear microscopy had low sensitivity of around 0-40% on extra pulmonary samples and lead to higher false negative rates. [96] In case of smear positive cases, the sensitivity of Xpert MTB/RIF when compared with culture was reported to be 90.5% in our study. In smear negative EPTB, the sensitivity and specificity as reported to be 97.2% and 99.5% respectively. Compared to another study done in Pakistan, which described sensitivity of 80% and specificity of 96% in smear negative cases, our study showed higher sensitivity and specificity. [88]

We report a pooled sensitivity of 63.5% and specificity of 99.5% for Xpert MTB/RIF when composite gold standard (Solid/liquid culture, Clinical features, Radiology and Histopathology results) is used which is similar to the study conducted in Vellore, which shows a sensitivity of 62% and specificity of 100%. [86] A study conducted in New Delhi describes a higher sensitivity of 91% and specificity similar to our study (99%). [87] Another work done in Mumbai showed a sensitivity and specificity of 80.6% and 99.6% respectively. [78]

A study done by Tortoli in Italy also showed 81.3% sensitivity which is higher compared to our study and 99.8% specificity which is almost similar to our study. [91] The composite gold standard may vary from study to study mainly based on the sample type and available diagnostic modalities, which may be the reason for the discrepancy between South Indian and North Indian studies.

For CSF, pleural fluid, tissue, pus and urine samples the sensitivity and specificity of Xpert MTB/RIF against solid/liquid culture was 100% individually, whereas for lymph node 92.3% and 100% were the sensitivity and specificity in our study. A good sensitivity of 86-100% was seen in specimens such as synovial fluid, pericardial fluid, peritoneal fluid, pus and fine needle aspirates in a study conducted in Mumbai. [78] Another work done by Pandey S showed a sensitivity of 100% for CSF, LN aspirates and tissue specimens and specificity of 100% for CSF and pleural fluid samples whereas 83% and 67% were the sensitivity and specificity of pus samples. And for other fluids they reported a sensitivity of 100% and specificity of 83%. [93]The positive and negative predictive value of CSF, pleural fluid, tissue, pus and urine samples when compared with culture, were reported to be 100% in our study which is similar to a study done in Pakistan.[88] In another published work done in New Delhi, India, the positive and negative predictive values were 83% and 97% , 82% and 83% and 100% and 96% for CSF, pleural fluid and urine samples respectively, which is lower when compared to our study.[87]

We report a lower sensitivity of 92.3% and specificity of 100% in lymph node specimens when compared with liquid culture. Study conducted in New Delhi, India showed a sensitivity and specificity of 88% and 91% respectively in lymph node

specimens. [87] Lower sensitivity and specificity of around 63% and 33% respectively were reported in lymph node tissues in another work done in Australia, which is low when compared to our study. [93]

Multidrug resistant tuberculosis is resistant to isoniazid and rifampicin. As per WHO, globally 490,000 people are estimated to have become multidrug resistant tuberculosis and 110,000 of rifampicin resistance in 2016. This multidrug resistance accounts for about 4.1% of new tuberculosis cases. Around 240,000 deaths were reported in 2016 due to multidrug resistant tuberculosis/ rifampicin resistant tuberculosis. [66]

In the present study, we report 5.35% of rifampicin resistance (3/56) indicating multidrug resistant tuberculosis. In a study done in India by Avashia, reported 5.4% (6/111) of rifampicin resistance by Xpert MTB/RIF, which is consistent with our study. [94] Similar study was also done by Gupta S et al in which rifampicin resistance was detected in 5.8% (5/85) of extra pulmonary samples. [95]

Xpert MTB/RIF test is a major advance in TB diagnostic testing, but has limitations, including the limited shelf-life of the diagnostic cartridges, some operating temperature and humidity restrictions, requirement for electricity supply, unknown long-term robustness, and the need for annual servicing and calibration of each machine.

Summary

SUMMARY

- The study was conducted to evaluate the role of Gene Xpert in diagnosis of extra pulmonary tuberculosis in tertiary care centres.
- Extra pulmonary samples from 500 patients who were suspected of extra pulmonary tuberculosis were collected.
- Out of 500 samples, 20 samples were from HIV positive patients.
- Most of the samples were from male patients (60%).
- Majority of samples were from patients under the age group 51-60 years (99 patients i.e. 19.8%).
- As per Kuppuswamy socioeconomic status, 51.8% and 33.9% of patients with extrapulmonary tuberculosis belonged to upper lower class (IV) and lower class (V) respectively.
- Maximum extra pulmonary samples obtained were CSF (33.4%**)** followed by pleural fluid (21.8%).
- Among the 500 extra pulmonary samples, only 56 samples were positive for *Mycobacterium tuberculosis* by Gene Xpert MTB/RIF.
- Around 80% of HIV patients had extra pulmonary tuberculosis.
- Among the 56 samples detected positive by Gene Xpert MTB/RIF, maximum number of positivity was seen between the age group 21-30 years (30.4%).
- Among the 500 extra pulmonary samples, 21 samples were positive by smear microscopy, 57 samples were positive for *Mycobacterium tuberculosis* by liquid culture (MIGIT), 56 samples were positive by solid culture (LJ medium)

and 56 samples were positive for *Mycobacterium tuberculosis* by Gene Xpert MTB/RIF.

- When compared with smear microscopy, Gene Xpert MTB/RIF showed a sensitivity of 90.5% and a specificity of 92.3%.
- The positive and negative predictive value of Gene Xpert when compared with smear microscopy was around 33.9% and 99.5% respectively.
- Smear microscopy detected Mycobacterium tuberculosis in 2 samples which were negative by Gene Xpert MTB/RIF.
- Gene Xpert MTB/RIF when compared with solid culture showed a sensitivity of 94.6% and specificity of 99.3%.
- The positive and negative predictive values of Gene Xpert MTB/RIF when compared with solid culture were 94.6% and 99.3% respectively.
- Gene Xpert MTB/RIF showed a sensitivity of 94.7% and specificity of 99.5% when compared with liquid culture.
- When compared with liquid culture, Xpert MTB/RIF showed 96.4% and 99.3% positive and negative predictive values respectively.
- Among the 56 positive samples detected by Gene Xpert MTB/RIF, 2 samples did not grow *Mycobacterium tuberculosis* in culture.
- Three culture positive samples were not detected in Gene Xpert MTB/RIF.
- Gene Xpert MTB/RIF, when compared with Composite Gold Standard showed 63.5%, 99.5%, 96.4% and 93% of sensitivity, specificity, positive predictive value and negative predictive value respectively.

- In smear negative cases, the sensitivity and specificity of Xpert MTB/RIF were 97.2% and 99.5% respectively.
- In case of CSF, pleural fluid, tissue, pus and urine specimens, the Xpert MTB/RIF showed 100% sensitivity and specificity when compared with both solid and liquid culture.
- About 100% sensitivity and specificity was shown by Xpert MTB/RIF when compared with solid culture in lymph node specimens.
- In case of lymph node specimens, 92.3% and 100% were the sensitivity and specificity of Xpert MTB/RIF when compared with liquid culture.
- Three extra pulmonary samples showed Rifampicin resistance by gene xpert MTB/RIF, indicating MDR tuberculosis incidence of 5.35% (3/56).

  Rifampicin resistance was observed in 11.1% of pus samples and 10% of pleural fluid samples.

Conclusion

CONCLUSION

The Xpert MTB/RIF assay is a new test that is revolutionizing tuberculosis (TB) control by contributing to the rapid diagnosis of TB disease and drug resistance. Major advantages of the Xpert MTB/RIF assay are that results are available quickly, and minimal technical training is required to run the test. Additionally, the Xpert MTB/RIF assay can quickly identify possible multidrug-resistant TB. From our study we conclude that Gene Xpert MTB/RIF is simple and reliable technique for diagnosing extra pulmonary tuberculosis with high sensitivity and specificity not only in smear positive cases but also in smear negative cases. It is a game changer not only in pulmonary tuberculosis control but probably also in extra pulmonary tuberculosis.

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Appendix

ABBREVIATIONS

ADA - Adenosine Deaminase

ATT - Anti Tubercular Treatment

BCG - Bacille Calmette Guerin

CBNAAT - Cartridge Based Nucleic Acid Amplification Test

CFP -10- Culture Filtrate Protein 10

CGS- Composite Gold Standard

CMI - Cell Mediated Immunity

CSF - Cerebro Spinal Fluid

DNA - Deoxyribo Nucleic Acid

ELISA - Enzyme Linked Immuno Sorbent Assay

EPTB - Extra Pulmonary Tuberculosis

ESAT-6 - Early Secretory Antigenic Target 6

HIV - Human Immuno Deficiency Virus

IFN- γ – Intereferon Gamma

IL-12 - Inter Leukin 12

LAMP - Loop Mediated Amplification

LAM - Lipoarabinomannan

LED - Light Emitting Diode

LJ - Lowenstein Jensen

LPA - Line Probe Assay

MDR -TB - Multi Drug Resistant Tuberculosis

MGIT - Mycobacterial Growth Indicator Tube

MTB - Mycobacterium tuberculosis

NTM - Non Tuberculous Mycobacteria

PANTA -Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin

PAS - Para Amino Salicylic Acid

PCR - Polymerase Chain Reaction

PPD - Purified Protein Derivative

RIF - Rifampicin Resistance

TLR -2- Toll like Receptor -2

TNF - Tumour Necrosis Factor

WHO - World Health Organization

PREPARATION OF REAGENTS AND MEDIA

1. ZIEHL NEELSEN STAIN:

Carbol fuchsin:

Basic fuchsin powder	1.6 gms
Phenol liquefied	22.5 ml
Alcohol (95%)	50 ml
Distilled water	422.5 ml

Dissolve the fuchsin in alcohol using a motor and pestle. Mix phenol with water and add to the dissolved dye. Filter the mixture before use.Warm phenol at 45^0 C in a water bath and measure with a warm pipette.

3% hydrochloric acid alcohol:

Concentrated hydrochloric acid	3 ml
Absolute alcohol	97 ml

Sulphuric acid:

20-25% of sulphuric acid in water (v/v) (acid must be added to water)

Loefflers methylene blue:

Methylene blue	0.2 gms
Absolute alcohol	10 ml
Distilled water	90 ml

Dissolve the dye in alcohol and then add water. Filter through a filter paper.

2. LOWENSTEIN- JENSEN MEDIUM:

Mineral salt solution:

Potassium dihydrogen phosphate	2.4 gm
Magnesium sulphate	0.24 gm
Magnesium citrate	0.6 gm
Asparagine	3.6 gm
Glycerol	12 ml
Distilled water	600 ml
Malachite green solution 2 %	20 ml
Egg solution	1000 ml

Dissolve the ingredients of mineral salt solution by heating.Autoclave at 121^0 c for 20 minutes to sterilize. This solution keeps indefinitely and may be stored in suitable amounts at 4^0c.

Prepare a 2% solution of malachite green in sterile water with sterile precautions by dissolving the dye in the incubator for 1-2 hours. This solution can be stored indefinitely and should be shaken before use.

Eggs must be fresh (not more than 4 days old). Wash them carefully with soap, warm water and brush, rinse in running water for 30 minutes. Drain the water and place the eggs in a sterile tray, cover with a sterile paper and dry till next day. Alternatively, eggs may be dried by cleaning the shell with methylated spirit and burning it off.

Scrub hands with soap and water, dry with spirit and then crack the egg with sterile knife and take the contents into a sterile beaker. Beat the egg with sterile egg beater.

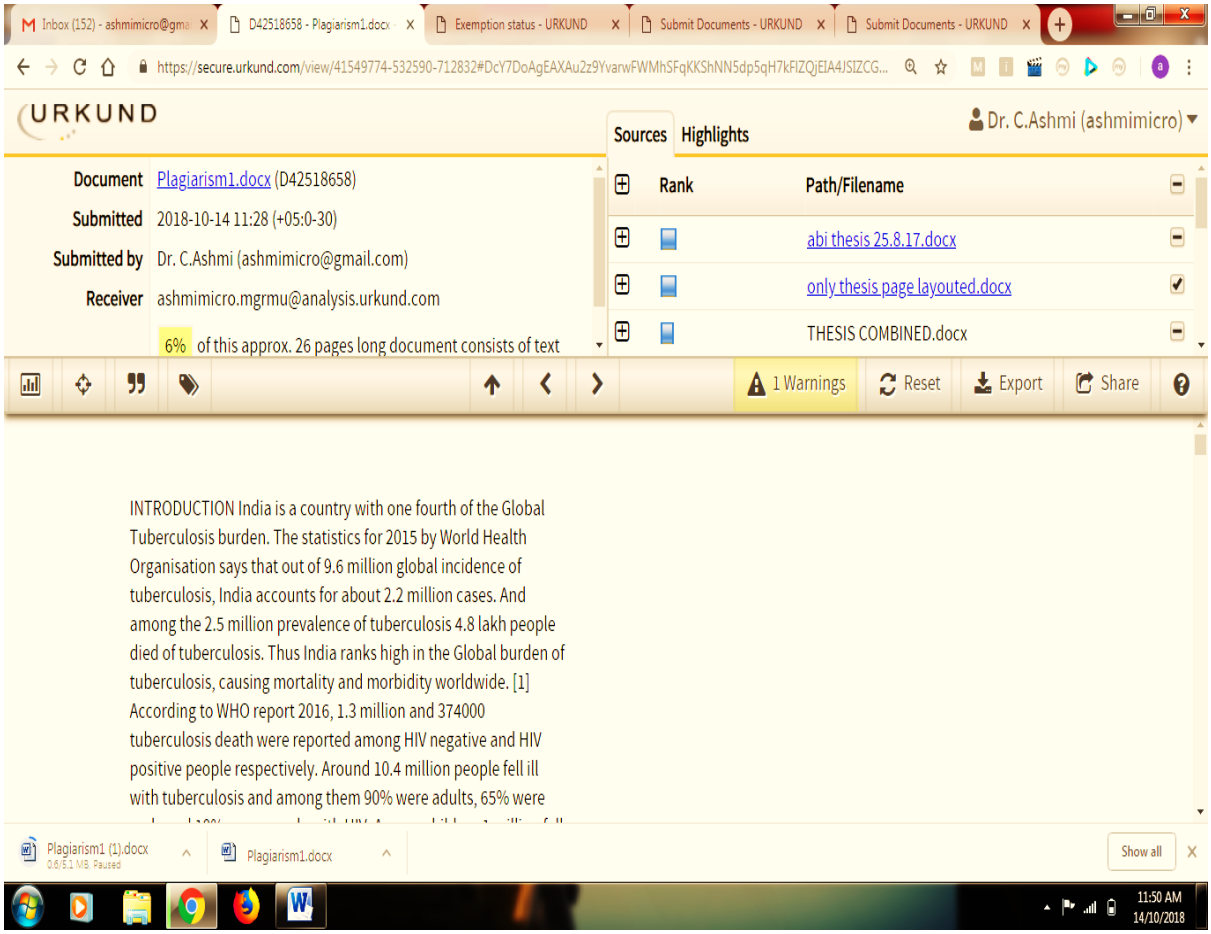
Mineral salt solution	600 ml
Malachite green solution	20 ml
20-22 hens egg	1000 ml

Distribute in 5 ml amounts in sterile screw capped tubes or McCartney bottles and screws the caps tightly on.Lay the tubes/ bottles on their sides and inspissate at 80- 85^0 c for 50 minutes.Leave the medium in the inspissator overnight and expose the media to 80- 85^0 c for an additional 30 minutes.

3. PREPARATION OF 1% CPC- NACL:

1 gram of cetylpyridinium chloride and 2 grams of sodium chloride are dissolved in 100 ml of sterile distilled water and distributed in 5 ml aliquots in sterile MacCartney bottles. The stock solution should be stored in dark coloured bottles at room temperature.

Annexures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INTRODUCTION India is a country with one fourth of the Global Tuberculosis burden. The statistics for 2015 by World Health Organisation says that out of 9.6 million global incidence of tuberculosis, India accounts for about 2.2 million cases. And among the 2.5 million prevalence of tuberculosis 4.8 lakh people died of tuberculosis. Thus India ranks high in the Global burden of tuberculosis, causing mortality and morbidity worldwide. [1] According to WHO report 2016, 1.3 million and 374000 tuberculosis death were reported among HIV negative and HIV positive people respectively. Around 10.4 million people fell ill with tuberculosis and among them 90% were adults, 65% were
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https://microbeonline.com/genexpert-mtbrif-assay-principle-procedure-results-interpretations/

Instances where selected sources appear:

17

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
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Dr C Ashmi
Postgraduate
Department of Microbiology
Guide: Dr B Appalaraju
PSG IMS & R
Coimbatore

Ref: Project No.17/242

Date: August 29, 2017

Dear Dr Ashmi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 02.08.2017 to conduct the research study entitled "*Evaluation of role of Xpert MTB/RIF in diagnosis of Extra Pulmonary Tuberculosis in patients attending tertiary care hospital*" during the IHEC meeting held on 18.08.2017.

The following documents were reviewed and approved:

1. Project submission form
2. Study protocol (Version 1 dated 02.08.2017)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 02.08.2017)
6. Current CVs of Principal investigator, Co-investigator
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 18.08.2017 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr R Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr S Shanthakumari	MD	Pathology	Female	Yes	Yes
4	Dr Sudha Ramalingam	MD	Epidemiologist Alt. member-Secretary	Female	Yes	Yes
5	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee